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**Regulation of Adrenal Corticosteroidogenesis: the role of
microRNAs in the control of aldosterone synthase and
11 β -hydroxylase expression.**

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the University of Glasgow

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Summary

Hypertension is a major risk factor for all cardiovascular disease, which is the largest known cause of global mortality. Essential hypertension—that is hypertension of unknown cause—is thought to have genetic and environmental risk factors. The best studied genetic system is that concerning corticosteroid biosynthesis. In humans, the principal glucocorticoid is cortisol, the main function of which is the control of intermediary metabolism; the major mineralocorticoid is aldosterone, which affects electrolyte and acid-base homeostasis. These steroid hormones are produced in the adrenal cortex through a series of biosynthetic reactions and under the influence of multiple regulatory factors. The final step in cortisol and aldosterone production involves, respectively, the cytochrome p450 enzymes, 11 β -hydroxylase and aldosterone synthase. These are encoded by the *CYP11B1* and *CYP11B2* genes which have a similar sequence and are highly polymorphic and lie, in tandem, on human chromosome 8.

Regulation of *CYP11B1* and *CYP11B2* mRNA abundance and of aldosterone and cortisol production have been extensively investigated. These studies have identified that there are several polymorphisms located across the locus which are associated with an increased aldosterone to renin ratio (ARR; used as an indicator of aldosterone regulation), inefficient 11 β -hydroxylation and essential hypertension. However, to date, no underlying mechanism for these associations has been established. Regulation of expression by transcription factors has been widely studied but, in this thesis, it is the role of a novel regulator, microRNA (miRNA) that is central.

miRNAs are short, non-coding RNAs which negatively regulate mRNA abundance. They are transcribed from endogenous loci, then undergo a series of enzymatic maturation reactions that result in the production of a single-stranded molecule of approximately 20 nucleotides. They function by associating with a group of proteins known as the RNA-induced silencing complex (RISC) and targeting the 3' untranslated region (3'UTR) of specific target mRNAs which they bind with imperfect complementarity. There are approximately 1100 human miRNAs, which have been implicated in the regulation of a range of target mRNAs and in

several pathologies including cancer and cardiovascular disease. The aim of this project was to investigate what role, if any, miRNAs have in the regulation of *CYP11B1* and *CYP11B2* expression and in corticosteroid production.

The studies in [Chapter 3](#) investigated miRNA regulation of corticosteroidogenesis in the adrenal cell line H295R. miRNA levels were universally reduced by targeting Dicer mRNA, a key component of the miRNA synthetic pathway, with short interfering RNA (siRNA). This study identified all of the CYP450 enzymes of the corticosteroidogenic pathway (CYP11A1, CYP17A1, CYP21A1, CYP11B1 and CYP11B2) as likely candidates for miR-mediated regulation based on mRNA and steroid analysis. The study also suggested that StAR, 3 β HSDII and 11 β HSDII are not modulated by miRNAs. To determine whether apparent miRNA regulation of *CYP11B1* and *CYP11B2* expression occurs by direct action at their 3'UTRs, reporter constructs were generated and tested. Under both basal and stimulated (AngII) conditions, these studies support a regulatory mechanism involving the 3'UTR of *CYP11B1* and *CYP11B2*. This chapter therefore provides evidence for miRNA-mediated regulation of corticosteroidogenesis.

In [Chapter 4](#), putative miRNA target sites in the *CYP11B1* and *CYP11B2* 3'UTR were identified using bioinformatic prediction algorithms and the miRNA expression profile of the normal human adrenal, as determined by microarray analysis. Based on miRNA target site prediction and analyses of the 3'UTR sequences (including such parameters as relative length, predicted sequence conservation and RNA secondary structure), *in silico* methods indicated the possibility that miRNAs can target *CYP11B1* and *CYP11B2* mRNA. Furthermore, the expression of 107 miRNAs in the normal adrenal gland was confirmed. Cross-referencing of microarray expression and bioinformatic data identified 16 adrenal miRNAs predicted to bind putative sites in *CYP11B1* and 16 predicted to bind *CYP11B2*; 12 of these miRNAs were common to both genes.

These formed the basis of the miRNA target validation studies in [Chapter 5](#). Sixteen adrenal miRNAs identified by bioinformatic analysis were tested individually *in vitro*. This was achieved by measuring mRNA expression, steroid production and 3'UTR reporter construct activity following artificially induced increases or reductions in the levels of specific miRNAs. These studies identified some miRNAs as being false positive predictions, while certain others were

validated. The miRNA that gave the most striking and consistent results, for targeting both *CYP11B1* and *CYP11B2*, was miR-24, which significantly decreased mRNA levels and steroid production. Analysis of adrenal miRNAs predicted only to target the *CYP11B2* 3'UTR confirmed miR-125a-5p and miR-125b as novel regulators, although effects on steroid secretion remain to be assessed. The studies in this chapter are the first to report of miRNA-mediated regulation of *CYP11B1* and *CYP11B2* expression.

Finally, in Chapter 6, the miRNA expression profiles of four aldosterone-producing adenoma (APA) samples were generated and compared to those of normal adrenal gland. Analysis identified 67 miRNAs expressed within the APAs; 54 were also present in the normal tissue. The levels of several miRNAs, including miR-24 and miR-125a-5p, were shown to be differentially expressed between the tissue types. This chapter also describes polymorphisms within the 3'UTR of the *CYP11B1* gene, generated from 26 normotensive patients. No novel SNPs were identified, but three are located in putative miRNA-binding sites. Previously, sequence analysis of the *CYP11B2* 3'UTR had been used to map miRNA binding sites, this identified two miRNA-binding sites which mapped to a known SNP. Taken together, the studies in this chapter provide a foundation for exploring altered miRNA function and/or expression within the adrenal gland.

In summary, the results presented in this thesis support a role for miRNA-mediated regulation of corticosteroidogenesis through actions on *CYP11B1* and *CYP11B2* expression. It demonstrates that miRNA are present in the adrenal gland, that miRNA-binding sites are present on the 3'UTR of relevant mRNAs, and that miRNAs are capable of post-transcriptional regulation that significantly alters mRNA abundance and steroid production. My findings describe a novel regulatory mechanism of corticosteroidogenesis. Whether this mechanism is altered in diseases such as essential hypertension remains to be elucidated. If so, miRNAs could, in the longer term, be used as targets for novel therapies or as biomarkers to classify more precisely specific pathologies.

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Publications

Abstracts and Awards

Wood S, Forbes GH, MacKenzie SM, Connell JMC, Davies E (2009) "MicroRNA: a novel post transcriptional regulator of 11 β -hydroxylase (*CYP11B1*) and aldosterone synthase (*CYP11B2*)". Endocrine Abstracts (19) P321

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Declaration

I declare that the work presented in this thesis is, to the best of my knowledge and belief, original and my own work, unless otherwise stated in the text. This work has not been submitted previously for a higher degree. It was carried out under the supervision of Professor Eleanor Davies and Dr Scott MacKenzie in the institute for Cardiovascular and Medical Sciences.

Stacy Wood
June 2011

Definitions/Abbreviations

| | |
|--------------------------------|---|
| * strand | Passenger strand/non functional |
| [K ⁺] _e | Extracellular potassium ion concentration |
| 11βHSDII | 11β-hydroxysteroid dehydrogenase type 2 |
| 12-HETE | 12-Hydroxyeicosatetraenoic acid |
| 12-LO | 12-lipoxygenase |
| 17-OH-P | 17-Hydroxyprogesterone |
| 17-OH-PREG | 17-Hydroxypregnenolone |
| 18-OHB | 18-hydroxycorticosterone |
| 18-OH-B | 18-Hydroxycorticosterone |
| 18-OHDOC | 18-hydroxy-11-deoxycorticosterone |
| 19-OHDOC | 19-hydroxy-11-deoxycorticosterone |
| 3' UTR | 3 prime untranslated region |
| 3β-HSD | 3β-hydroxysteroid dehydrogenase |
| 5' UTR | 5 prime untranslated region |
| A | Adenosine |
| A'dione | Androstenedione |
| ACE | Angiotension Converting Enzyme |
| AD1- AD6 | Adrenal transcription factor binding site 1 - 6 |
| ADARs | Adenosine deaminase action on RNA |
| AGO | Argonaut Protein |
| AGT | Gene encoding angiotensinogen |
| AIP | Aldosterone-induced Proteins |
| AKR1C3 | Aldo-keto-reductase family |
| Aldo | Aldosterone |
| AME | Apparent Mineralocorticoid Excess |
| AngII | Angiotensin II |
| ANOVA | Analysis of variance |
| AP-1 | Activation protein-1 |
| APA | Aldosterone-producing adenoma |
| AP-O | Aminopeptidase-O |
| AR | Androgen receptor |
| ARE | Adenylate Uridylate-rich element |
| ARR | Aldosterone to renin ratio |
| AT1R | Angiotensin II receptor type I |
| AT2R | Angiotensin II receptor type II |
| ATCC | American Type Culture Collection |
| ACTH | Adrenocorticotrophic hormone |
| ATF | Activating transcription factors |
| ATP | Adenosine triphosphate |
| AU | Arbitrary units |
| B | Corticosterone |
| BCA | Bicinchoninic acid |
| bp | Base pairs |
| BRIGHT | British Genetics of Hypertension |
| BSA | Bovine serum albumin |

| | |
|-------------------|---|
| C | Cholesterol |
| Ca ²⁺ | Calcium ions |
| CAH | Congenital adrenal hyperplasia |
| Calmodulin or CaM | Calcium-modulating protein |
| CaMK | CaM-dependent protein kinases |
| cAMP | Cyclic adenosine monophosphate |
| CAT-1 | Cationic amino acid transporter |
| CCD | Cortical collecting duct |
| cDNA | Complementary DNA |
| c-fos | Protein product of FOS gene |
| CHARGE | Cohorts for Heart and Ageing Research in Genome Epidemiology BP consortium |
| CHIF | Channel-inducing factor |
| CLL | Chronic lymphocytic leukaemia |
| CMV | Cytomegalovirus |
| CNS | central nervous system |
| CO | Cardiac output |
| CRE | cAMP response element |
| CREB | CRE-binding protein |
| CRH | Corticotrophin-releasing hormone |
| Ct | Cycle threshold |
| C-terminus | Carboxyl terminal domain |
| CVD | Cardiovascular diseases |
| CYP11B1 | 11 β -Hydroxylase |
| CYP11B2 | Aldosterone Synthase |
| CYP17A1 | 17 α -Hydroxylase |
| CYP450s | Cytochrome P450 enzymes |
| DAG | Diacylglycerol |
| Dcp2 | Decapping enzyme 2 |
| DGCR8 | Drosha and Di George critical region 8 |
| DHEA | Dehydroxyprogesterone |
| DHFR | Dihydrofolate reductase |
| DLRA | Dual Luciferase Reporter Assay |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxynucleoside triphosphates |
| DOC | 11-Deoxycorticosterone |
| dsRBD | Double stranded RNA binding domain |
| dsRNA | Double-stranded RNA |
| E | Cortisone |
| EDTA | Ethylenediamine tetra-acetic acid |
| eIF | Eukaryotic initiation factor |
| ENaC | Epithelial sodium channel |
| ER | Estrogen receptors |
| ERK | Extracellular signal-regulated kinase |
| ET-1 | Endothelin-1 |

| | |
|----------------|---|
| F | Cortisol |
| FAM | Fluorescein |
| FCS | Fetal calf serum |
| FFPE | Formalin-fixed Paraffin-embedded |
| FH | Familial hyperaldosteronism |
| FOS | Gene encoding c-fos |
| g | G-force (relative centrifugal force) |
| G | Guanine |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GILZ | Glucocorticoid-induced leucine-zipper |
| GPCR | G-protein coupled receptors |
| GR | Glucocorticoid receptor |
| GRA | Glucocorticoid Remediable Aldosteronism |
| GRE | Glucocorticoid-responsive elements |
| GTP | Guanosine-5'-triphosphate (GTP) |
| GW182 | Glycine(G)-tryptophan(W) protein of 182 kD |
| GWAS | Genome-wide association study |
| HDL | High-density lipoproteins |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HITS-CLIP | High throughput sequencing of cross-linking immunoprecipitation |
| HMGA2 | Gene encoding High-mobility group AT-hook 2 |
| HPA | Hypothalamic-pituitary-adrenal |
| HPLC | High-performance liquid chromatography |
| HRE | Hormone-response elements |
| hsp | Heat shock protein |
| HWE | Hardy-Weinberg equilibrium |
| IC | Intron conversion |
| IHA | Bilateral idiopathic hyperplasia |
| INTERSALT | International Study of Salt and Blood Pressure |
| IP3 | 1,4,5-trisphosphate |
| IRES | Internal ribosome entry site |
| ITS | Insulin-transferrin-selenium |
| IU | International Unit |
| K ⁺ | Potassium ions |
| kb | Kilobases |
| kcal/mol | Kilocalorie per mole |
| Kd | Dissociation Constant |
| kD | KiloDalton |
| KiRasA | GTP-dependent signalling protein K-ras2 |
| KIT | Gene encoding tyrosine-protein kinase Kit |
| LAR II | Luciferase assay buffer II |
| LB | Luria Broth |
| LC/MS:MS | Liquid chromatography tandem mass spectrometry |
| LD | Linkage disequilibrium |
| LDL | Low-density lipoproteins |
| LNA | Lock Nucleic Acid |
| Lod | Logarithmic odds ratio |

| | |
|-------------------|---|
| LPP | Gene encoding lipoma-preferred partner is a protein |
| MAF | Minor allele frequency |
| MFE | Minimum free-energy |
| miRISC | miRNA-Induced silencing complex |
| miRNA | Micro ribonucleic acid (microRNA) |
| mM | Millimolar |
| mm | Millimetre |
| MONICA | North Glasgow Monitoring of Trends and Determinants in CVD |
| MR | Mineralocorticoid receptor |
| mRNA | Messenger ribonucleic acid |
| MTHFR | Gene encoding Methylenetetrahydrofolate reductase |
| NaOAc | Sodium acetate |
| NAPDH | Nicotinamide adenine dinucleotide phosphate |
| NBRE-1 | Neuronal growth factor-induced clone B response element |
| Nedd4-2 | Neural precursor cell expressed, developmentally down-regulated 4?2 |
| nGRE | Negative glucocorticoid-responsive elements |
| NGRI-B | Nneuronal growth factor-induced clone B |
| NHE | Na ⁺ /H ⁺ exchanger |
| nm | Nanometer |
| nM | Nanomolar (nanomoles per litre) |
| NR3C2 | Gene encoding mineralocorticoid receptor |
| N-terminus | Amino acid terminal domain |
| Obs. Het | Observed Heterozygosity |
| P | Progesterone |
| p16 | Cyclin-dependent kinase inhibitor 2A |
| PA | Primary Aldosteronism |
| PABP | Poly A binding protein |
| PACT | Protein activator of PKR |
| PAZ | Piwi-Argonaute-Zwille |
| P-bodies | Processing-bodies |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| pEZX | Reporter construct |
| pg | Picogram |
| piwiRNA | P-element-induced wimpy testis RNA |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PKR | Interferon-induced protein kinase |
| PLB | Passive Lysis Buffer |
| POMC | Pro-opiomelanocortin |
| PR | Progesterone receptor |
| PREG | Pregnenolone |
| pre-miRNA/pre-miR | Preliminary miRNA |
| pri-miRNA/pri-miR | Primary miRNA |
| PTEN | Phosphatase and tensin homologue |
| PTK9 | Gene encoding |
| qRT-PCR | Quantitative Real-time Polymerase chain reaction |

| | |
|--------------|---|
| QTL | Quantitative trait loci |
| RALES | Randomized Aldactone Evaluation Study |
| Ran-GTP | RAs-related Nuclear protein-Guanosine-5'-triphosphate |
| RAS | Renin-Angiotensin System |
| REST | Repressor element 1-silencing transcription factor |
| RIN | RNA integrity number |
| RISC | RNA induced silencing complex |
| RLB | Reporter Lysis Buffer |
| RLU | Relative light units |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| rpm | Revolutions per minute |
| RT | Reverse transcriptase |
| S | 11-Deoxycortisol |
| SEM | Standard error of the mean |
| SF-1 | Steroidogenic factor-1 |
| SGK1 | Serum- and glucocorticoid-regulated kinase 1 |
| SILAC | Stable isotope labelling with amino acids in cell culture |
| siRNA | Short interfering ribonucleic acid |
| SLIT2 | Gene encoding Slit homolog 2 protein |
| SNP | Single nucleotide polymorphism |
| SPRI | Solid phase reversible immobilization |
| ssRNA | Single stranded RNA |
| StAR | Steroidogenic Acute Regulatory |
| SV40 | Simian virus 40 |
| T | Thymine |
| TASK channel | TWIK-related acid-sensitive potassium channel |
| TBE | Tris/Borate/EDTA |
| TFS | Transcription factors |
| THDOC | Tetrahydrodeoxycorticosterone |
| THS | Tetrahydrodeoxycortisol |
| TIMP3 | Tissue inhibitor of metalloproteinase 3 |
| TPR | Total peripheral resistance |
| TRBP | Tar RNA binding protein |
| tRNA | Transfer RNA |
| U | Unit |
| UPL | Universal ProbeLibrary |
| UTR | Untranslated Regeion |
| UV | Ultraviolet |
| V | Voltage |
| v/v | Volume/volume |
| WR | Working reagent |
| Wt | Wild-type |
| XRN-2 | 5'-3' exoribonuclease 2 |
| ZF | Zona fasciculata |
| ZG | Zona glomerulosa |
| ZR | Zona reticularis |
| μM | Micromolar (micromoles per litre) |

1 Introduction

High blood pressure and associated cardiovascular and cerebrovascular disease are massive contributors to morbidity and early mortality in developed societies. While lifestyle factors such as smoking, high saturated fat and high salt diets and reduced rates of physical exercise are important factors, variations in the efficiency of several physiological systems, often genetically-based, contribute significantly.

The adrenal cortex is among the most important of these. In the following review, the structure of the adrenal cortex and the nature of its principle products, the corticosteroids, are described. The synthesis and control of secretion of the two major categories of corticosteroids, those which affect electrolyte and acid-base homeostasis, the mineralocorticoids, and those whose main function is in the control of intermediary metabolism, the glucocorticoids, are briefly outlined. Their mechanism of action in target tissue is then described, addressing briefly classical receptor-linked processes and presenting information on the more recently discovered non-classical modes of action.

The importance of the adrenal cortex in hypertension will be discussed by describing the effects of corticosteroids in experimental animal models and in a group of relatively uncommon disorders of corticosteroidogenesis. However, the most frequent form of hypertension is primary or essential hypertension, that is, hypertension of unknown aetiology. Studies over recent decades strongly suggest that small, genetically-related differences in the control of secretion and rate of catabolism of corticosteroids may predispose to hypertension and cardiovascular diseases. These studies are outlined and evaluated.

In the control of metabolism, mRNA transcription is initiated in response to specific agonist-induced transcription factors. The resulting mRNA may be modified prior to the process of translation into new protein and the longevity and eventually destruction of mRNA is determined by other biochemical processes. The efficiency of each of these components may contribute to biological variation of clinical significance. In the final sections of this review, the structure and control of expression of two key genes in corticosteroid synthesis are described in detail. Of particular relevance to this thesis, the identification and action of novel RNA species, microRNAs, powerful modulators

of mRNA is analysed. It is with these microRNAs that the experimental work described in sections 3, 4, 5 and 6 is concerned.

1.1 Blood Pressure and Hypertension

The heart contracts periodically, with each beat it ejects a bolus of blood into the circulation. That creates a cyclic change in blood pressure throughout the vasculature. Blood pressure is highest immediately after the contraction, known as systolic blood pressure and lowest prior to the next contraction, known as diastolic blood pressure. Blood pressure can be defined as the cardiac output (CO, the volume of blood ejected from the heart) multiplied by the total peripheral resistance (TPR, the sum of resistance in vasculature), these are under independent control (Figure 1-1).

Blood pressure is maintained by a variety of homeostatic controls (Figure 1-1). These must tolerate everyday stresses such as changes in posture and exercise to ensure continuous perfusion of vital organs. Under healthy circumstances, normal adult blood pressure is defined as systolic pressure under 120 mmHg and diastolic pressure under 80 mmHg (Chobanian *et al.*, 2003) but this is arbitrary. This is further addressed in Section 1.1.2. Blood pressure is under the control of several complex regulatory systems including the sympathetic nervous system, endocrine stimuli, local tissue control (for example, flow-autoregulation) and finally regulation of sodium and water balance by the kidneys (Figure 1-1). The renal component is under the control of the renin-angiotensin system, which through the production of angiotensin II (AngII) regulates the synthesis of aldosterone and will be described in Section 1.1.1.

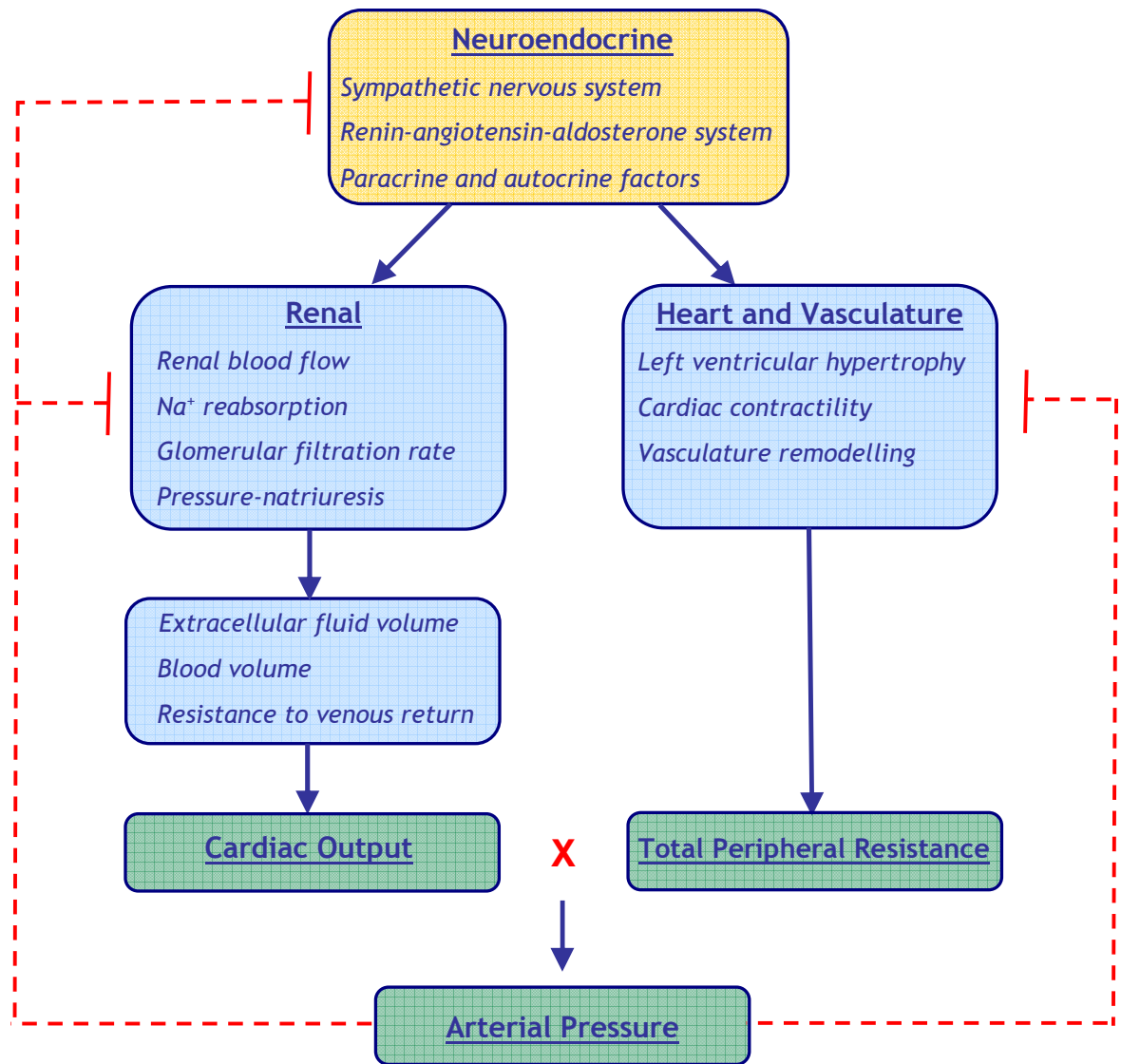


Figure 1-1. Mechanisms of Arterial Pressure Regulation.

Adapted from Cowley (2006).

1.1.1 The Renin-Angiotensin System (RAS)

The renin-angiotensin system (RAS) (Figure 1-2) is composed of a series of tightly-regulated enzymatic reactions, the ultimate product of which is Angiotensin II (AngII), a pressor octapeptide. Activation of the RAS cascade occurs when low blood pressure is detected by the renal afferent arteriole or when decreased NaCl concentration is sensed by specialised epithelial cells of the distal tubule called the macula densa, stimulating the secretion of renin from renal juxtaglomerular cells. Renin is an aspartyl protease which is originally synthesised from preprorenin, subsequently cleaved to prorenin and finally to renin by the prorenin processing enzyme. Renin catalyses the cleavage of angiotensinogen, a hepatic α -globulin to angiotensin I. N-terminal amino acids of angiotensin I, an inactive decapeptide, are cleaved by angiotensin converting enzyme (ACE) to produce the vasoactive, octapeptide AngII (Figure 1-2). ACE was first discovered in the 1950s and is a membrane-bound zinc metalloprotease located on vasculature endothelial surfaces in the lungs, the kidneys, the intestine and the placenta.

AngII has many actions in several target tissues including, the vasculature, the adrenal cortex, the kidney, the brain and adipose tissue. It binds to specific transmembrane G-protein coupled receptors (GPCR) of two types: type 1 (AT1R) or type 2 (AT2R) (Figure 1-2) (Timmermans *et al.*, 1993). Once activated, the different subtypes of receptors mediate opposing effects on resistant vessels, the AT1R promoting vasoconstriction and the AT2R receptor vasodilation. Both receptors have similar affinities for AngII, are widely distributed and yet have heterogeneous expression patterns. In human adults AT1R serves as the principle mediator of blood pressure regulation (Timmermans *et al.*, 1993; Shanmugam *et al.*, 1996). By acting on AT1R, AngII contributes to blood pressure regulation by three main actions: it promotes the release of aldosterone from the adrenal cortex, it increases the re-absorption of sodium in the distal convoluted tubules of the kidney and finally, it causes vasoconstriction.

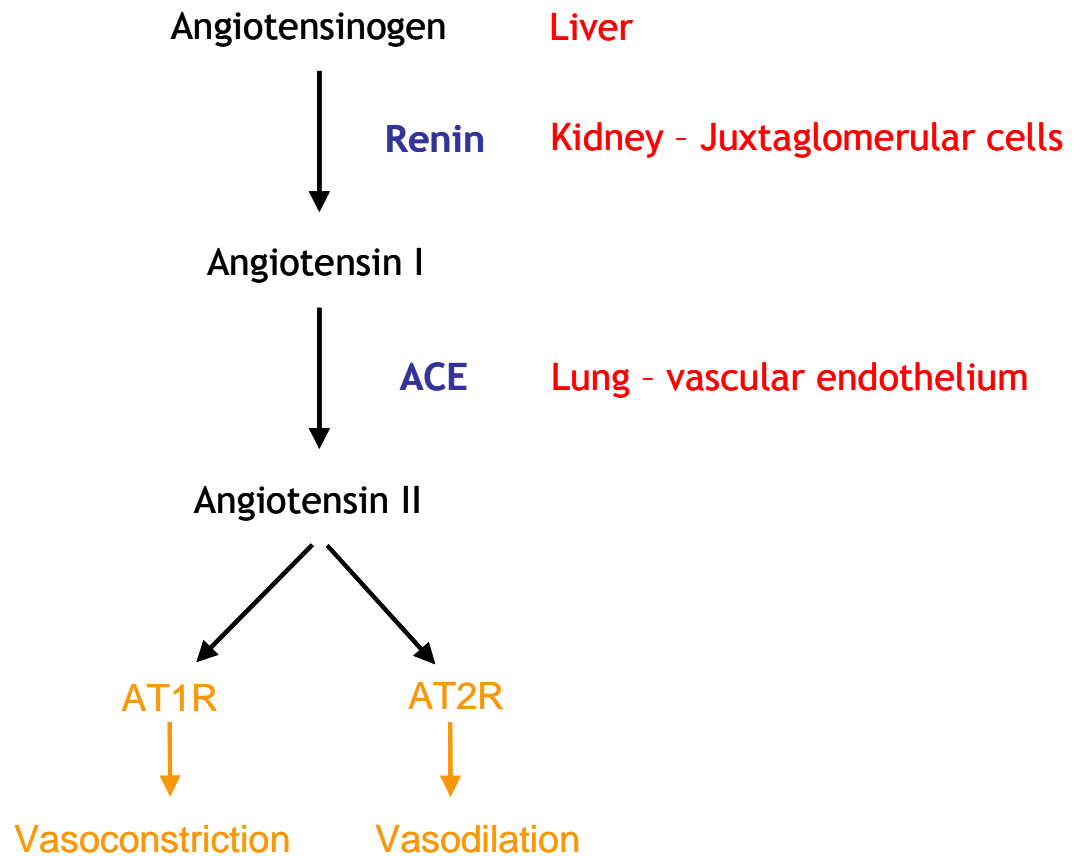


Figure 1-2. The Renin-Angiotensin System.

ACE: angiotensin converting enzyme; AT1R: angiotensin II receptor type 1; AT2R: angiotensin II receptor type 2.

1.1.2 Hypertension

Hypertension; that is, blood pressure above the upper limit of the normal range, is the major risk factor for all cardiovascular diseases (CVD); 54% of death from strokes and 47% of death in patients of ischemic heart disease can be attributed to hypertension (Lawes *et al.*, 2008). CVD is the largest known cause of mortality globally (Chobanian *et al.*, 2003).

Blood pressure is subject to short term variability and obtaining a 'representative' clinical value in clinic is difficult. Therefore, defining hypertension is problematic (Carretero and Oparil, 2000). As a necessary, though arbitrary, guide hypertension is defined as a systolic blood pressure greater than 140 mmHg or a diastolic blood pressure over 90 mmHg. However, risk varies incrementally with blood pressure (Chobanian *et al.*, 2003), even at levels below 140/90 mmHg ('pre-hypertensive') (Table 1-1) (Kshirsagar *et al.*, 2006). This emphasises that small changes in the factors governing blood pressure can have significant detrimental effects.

In only a small number of patients (approximately 5%) can an underlying medical cause of their hypertension be identified (Section 1.4.1.1). The remaining 95% are classified as having essential hypertension (also known as primary hypertension or idiopathic hypertension) (Section 1.1.3).

Table 1-1. Classification of Blood Pressure (Chobanian *et al.*, 2003).

| Classification | Systolic BP (mm Hg) | Diastolic BP (mm Hg) |
|----------------------|---------------------|----------------------|
| Normal | <120 | and <80 |
| Pre-hypertension | 120-139 | or 80-89 |
| Stage 1 Hypertension | 140-159 | or 90-99 |
| Stage 2 Hypertension | >160 | or >100 |

1.1.3 Essential Hypertension

The aetiology of essential hypertension remains elusive, despite large numbers of investigative studies. However, there are several known risk factors which contribute to the development of hypertension which can be split into lifestyle or genetic risk factors. The next section will briefly discuss the effects of these on blood pressure.

1.1.3.1 Lifestyle Risk Factors

Environmental risk factors include high sodium intake and low potassium intake, obesity, stress, lack of exercise, smoking and alcohol intake. Of these, sodium and potassium intake are the most relevant to the studies of this thesis because they alter the production and action of corticosteroids (Section 1.2.5 and 1.3).

The International Study of Salt and Blood Pressure (INTERSALT) project was a large, prospective epidemiological study which involved 52 centres from 32 countries. Results from 10,079 individuals identified a strong link between sodium excretion and systolic blood pressure that was independent of any other risk factors (Stamler *et al.*, 1989). Other intervention studies reduced salt intake in either normotensive or hypertensive people and showed that blood pressure was reduced on low sodium diets. This is true for both acute (less than 4 weeks) or prolonged salt restricted diets, which reduced salt intake from 10 g/day to 5 g/day (Macgregor *et al.*, 1982; Meneton *et al.*, 2005). Moreover, a recent meta-analysis of 19 separate cohorts demonstrated that high sodium intake (average increase in salt intake of 5 g/day) increased the risk of stroke and total incidence of CVD (Strazzullo *et al.*, 2009).

INTERSALT identified a relationship between potassium intake and blood pressure; urinary excretion of potassium had an independent, inverse association with systolic blood pressure. Also, the ratio of sodium: potassium excretion was positively and significantly related to both systolic and diastolic blood pressure (Stamler *et al.*, 1989). A meta-analysis of 33 randomised controlled trials (totalling 2609 subjects), which each investigated the use of potassium supplements, found that exogenous potassium significantly reduced blood pressure (Whelton *et al.*, 1997). Further, hypertensive animals fed high

potassium and high sodium diets had lower blood pressures than those on high sodium, standard potassium diets (Adroque and Madias, 2007). Corticosteroids both respond to, and participate in, the control of electrolyte status (Sections 1.2.5 and 1.3).

1.1.3.2 Genetic Risk Factors

The studies in this thesis relate to the control of gene transcription. It is therefore relevant briefly to consider how genetic variation and disease in the population is studied. Evidence implicating a genetic link to blood pressure regulation and pathology is strong and arises from several sources. Several key family studies suggest a genetic link; individual members of a family unit have a greater similarity in their blood pressures when compared to individuals from other families (Longini *et al.*, 1984). Findings from twin studies found a greater concordance of blood pressure in monozygotic twins than in dizygotic twins (Feinleib *et al.*, 1977). Furthermore, the blood pressures of biological siblings is more highly correlated than those of adopted siblings (Biron *et al.*, 1976; Rice *et al.*, 1989). Other evidence of a genetic link arises from several defined monogenic diseases which affect blood pressure (Section 1.4.1.4).

Heritability is a parameter measuring the likelihood that a trait is attributed to genetic rather than environment factors. Blood pressure has a relatively high percentage of heritability, reported 15-40% for systolic blood pressure and 15-30% for diastolic. A systematic review of family studies estimated the joint heritability rate to be approximately 35% (Ward, 1990). Thus, evidence supports a genetic risk to blood pressure.

Hypertension is a polygenic disorder, where the combination of several detrimental genetic traits is manifested as a small increase in blood pressure. However, to date, the identity of the genes responsible for this association remains elusive, although information obtained from the study of rare monogenic disorders has made a useful contribution to our understanding of the genetics of hypertension (Section 1.4.1.4). Also, several other scientific approaches such as linkage and association studies have identified causative genetic defects that may allow for more focused preventative measures and treatment.

Association Studies

Genome-wide association studies (GWAS) as the name suggest, span the entire genome and tend to involve the highest number of subjects as well as being the most expensive method to perform. GWAS assesses over a million single nucleotide polymorphisms (SNPs) located throughout the genome to identify variations important to a disease trait. GWAS studies are carried out in large populations, in cases and controls or in individuals. The aim is to identify novel associations of SNPs with diseases, and been useful in the study of many diseases (Burton *et al.*, 2007).

However, the complex nature of blood pressure, the problem of choosing appropriate controls and the inadequate coverage of SNPs in commercial gene arrays have prevented GWAS from being as successful for the investigation of hypertension. Of the twelve independent blood pressure GWAS studies (all of European origin), only two have successfully identified regions with positive associations to blood pressure: the Global BPGen consortium and the CHARGE (Cohorts for Heart and Ageing Research in Genome Epidemiology BP consortium) (Newton-Cheh *et al.*, 2009; Levy *et al.*, 2009). In total, 14 independent genetic loci have been identified for blood pressure traits, several of which lie near genes which encode enzymes (kinases and cyp450 (see Section 1.2.2.1)), solute channels, transcription factors, a cell signalling protein and a structural protein (Ehret, 2010). Two of the candidates, the CYP17A1 gene and the MTHFR gene are plausible candidates (their significance is explained in Section 1.4.1.4), the role and implication of the others remains to be understood. The analysis of this in GWAS data predicts that each associated allele would represent a change of about 1 mm Hg in systolic pressure (Ehret, 2010). Thus, genetic variations may each have a small impact on blood pressure but the coexistence of several detrimental loci could have a cumulative effect.

Linkage Analysis

Linkage analysis studies aim to identify genomic loci, known as quantitative trait loci (QTL) which are genetically linked, heritable and may be attributable to the disease being investigated. The largest study of this type has been the British Genetics of Hypertension (BRIGHT) study that identified two QTL (Caulfield *et al.*, 2003). However, the results have been criticised due to the location of the QTL being close to the end of chromosomes, this is often problematic as linkage programmes can generate errors at the extremes of chromosome, and the use of multiple testing which requires a higher Lod (logarithmic odds ratio, a score used to indicate the likelihood of linkage) of 3.6 to attain significance. In the case of BRIGHT the Lod score was only 2.5 (Binder, 2007; Delles *et al.*, 2010). Individual linkage studies have identified QTLs on nearly all human chromosomes but these are rarely replicated by other studies (Binder, 2007). Thus, linkage analysis is not an accurate or reproducible method for studying the importance of genes and hypertension.

Candidate Gene Analysis

An alternative approach, candidate gene studies, dissects the variability of genes with a known physiological link to blood pressure, such as those involved in salt and fluid homeostasis (e.g. RAS) or the adrenergic system. The results of these studies have been inconsistent, frequently due to poor study design (selection of heterogeneous cases and controls) or SNP coverage. One of the most extensively studied genes using this approach is the *AGT* gene which encodes angiotensinogen, a pivotal enzyme in the RAS (Figure 1-2). Many SNPs have been identified in this gene in coding and non-coding DNA regions. Two SNPs, T174M and M235T, are associated with hypertension in some studies but not others (Dickson and Sigmund, 2006). A meta-analysis of several studies failed to confirm these associations but the T allele at position 235 was associated with an increased relative risk of hypertension (Sethi *et al.*, 2003). Another candidate is the *ACE* gene, which again has a high frequency of polymorphisms, the one of most interest being a 287 base-pair insertion/deletion within intron 16. This polymorphism is associated with increased ACE plasma levels, coronary artery disease and endothelial dysfunction, as measured by monitoring cardiac blood flow following infusion of acetylcholine and sodium nitroprusside (Prasad *et al.*, 2000). However it does not play a role in hypertension (Jeunemaitre, 2008). The

genes that provided the focus in this thesis, *CYP11B2* and *CYP11B1*, are also well-studied candidate genes and will be discussed in greater detail in sections 1.2.2, 1.2.6 and 1.4.2.

To summarise, there are significant genetic and lifestyle components to blood pressure regulation and the development of hypertension. Essential hypertension is a complex disease and the genetic aetiology is difficult to study. One candidate system which studies have shown to be important for blood pressure regulation and the development of essential hypertension is the adrenal cortex. The main mineralocorticoid, aldosterone and, main glucocorticoid, cortisol are synthesised here and altered synthesis plays a role in the aetiology of essential hypertension. The next three sections of this review will focus on these compounds, their actions and the evidence of their dysregulation in several pathologies.

1.2 Corticosteroids

Steroid hormones are classified by their structural characteristics and biological function. Examples include estrogens, androgens, progestins, corticosteroids and vitamin D. All steroids are derived from cholesterol. Corticosteroids are products of the adrenal cortex and, based on their function, can be subdivided into mineralocorticoids and glucocorticoids, the main human examples of which are aldosterone and cortisol, respectively. The adrenal cortex also synthesises androgens and small quantities of estrogens.

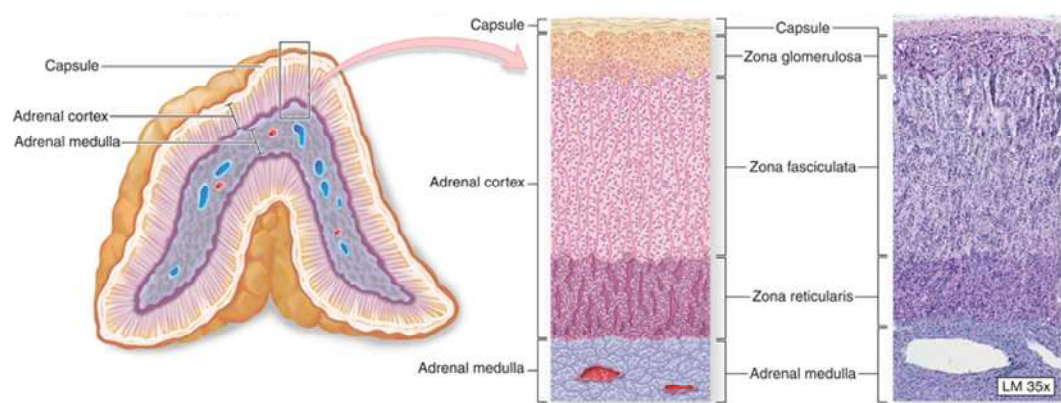
1.2.1 *The Adrenal Gland*

The adrenal glands are paired endocrine organs that lie anatomically superior to each kidney. Each adrenal gland has independent blood, lymph and nervous supplies and can therefore function independently of one another. The glands are surrounded by an outer fibrous capsule. The functional tissue of the adrenal gland is divided into two separate anatomical regions, which have specialised functions. The inner medulla is responsible for producing and secreting catecholamines. The primary function of the outer region, the adrenal cortex, is to produce and secrete steroid hormones, for example aldosterone and cortisol.

The adrenal cortex is divided into three zones, which are functionally and histologically distinguishable. The names, position and functions of each zone are outlined in Table 1-2 and illustrated in Figure 1-3.

Table 1-2. Name, Function and Location of the Layers of the Adrenal Cortex.

| Zone | Position | Steroids Produced |
|-----------------------|----------|----------------------------------|
| Zona glomerulosa (ZG) | Outer | Mineralocorticoids |
| Zona fasciculata (ZF) | Middle | Glucocorticoids |
| Zona reticularis (ZR) | Inner | Sex steroids and glucocorticoids |

**Figure 1-3. Cross-Sectional Illustration of the Layers of the Adrenal Gland.**

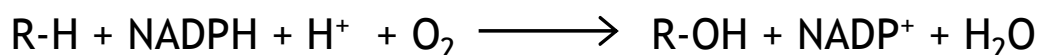
Adrenal gland showing inner medulla, outer cortex and capsule. Magnified section showing schematic and haematoxylin and eosin staining of individual layers (taken from the McGraw-Hill online learning centre; chapter 10 Endocrine System, <http://www.mhhe.com/biosci/ap/seeleyessentials/student/olc/chap10out.html>).

1.2.2 Enzymes of the Corticosteroid Pathway

Two classes of enzymes are required for corticosteroidogenesis: five cytochrome P450 enzymes and one hydroxysteroid dehydrogenase. Characteristics of each of these enzymes are briefly discussed below; the succeeding sections will describe the features and functions of these enzymes in relation to corticosteroid synthesis. Steroid synthesis involves a complex pathway of intermediates; the names and common abbreviations of components in the pathway are in Table 1-3 and their structures illustrated in Figure 1-4.

1.2.2.1 Cytochrome P450 Enzymes

To convert cholesterol to the C21 corticosteroids (Figure 1-4), most of its side chain must be removed and the pregnane structure modified by a series of oxidative reactions. Androgen synthesis requires the complete removal of the side chain. The majority of enzymes involved are mixed function oxidases or mono-oxygenases belonging to the cytochrome P450 group of enzymes (CYP450s). These are a large class of haemoproteins which catalyse oxidative reactions in a wide range of cells. They are so named because, when in a carbon-monoxide-bound form, they produce an absorption band at 450 nm. CYP450s are a very diverse family which is sub-classified into 69 families based on structural and phylogenetic similarities (Nelson *et al.*, 1996). CYP450s are involved in a wide range of cellular functions including xenobiotic metabolism, steroid synthesis and lipid synthesis. They catalyse the addition of an oxygen atom from a molecule of oxygen to the substrate in a locus and stereo-specific manner, utilising an electron donor, usually NADPH. The remaining oxygen atom is reduced to water. The stoichiometry of the reaction is;



1.2.2.2 Hydroxysteroid Dehydrogenases

Hydroxysteroid dehydrogenases are bidirectional, non-metalloenzymes that are expressed in several tissue types. There are several different isoforms of each dehydrogenase, each with a specific expression pattern, cellular localisation, catalytic activity and each is encoded by a different gene. All dehydrogenase enzymes utilise co-factors, which act as hydrogen donors or acceptors, for example $\text{NAD}^+/\text{NADP}^+$. Within the adrenal cortex, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) is essential for the synthesis of mineralocorticoids, glucocorticoids and androgens. In contrast, hydroxysteroid dehydrogenases expressed in peripheral tissue, for example in the kidney, are responsible for metabolism of steroids (Penning, 1997; Payne and Hales, 2004).

1.2.3 Cholesterol Transfer into the Mitochondria

Cholesterol is a sterol and is the precursor of all steroids Figure 1-4. It is comprised of four hydrocarbon rings, a hydrocarbon tail (carbons 20-27) and a 3 β -hydroxyl group. Cholesterol can be synthesised *de novo* in the adrenal cortex but most is derived from extra-adrenal sources, predominantly in the liver or the diet. Cholesterol is amphipathic and must be incorporated in low-density lipoproteins (LDL) or high-density lipoproteins (HDL) to circulate in the blood.

In humans, the adrenal gland preferentially uses cholesterol derived from LDL in circulating plasma, which is internalised into the adrenal cells by receptor-mediated endocytosis whereas, in rats the main source is from HDL (Gwynne and Strauss, 1982). Once inside the cell, cholesterol must be transported across the outer mitochondrial membrane. Several transporters have been identified which facilitate this transfer but it is now widely accepted that the principle transporter is the steroidogenic acute regulatory (StAR) protein. Some cholesterol can diffuse across the membrane but its hydrophobic nature means that the rate of diffusion is extremely slow and contributes little to steroidogenesis (Phillips *et al.*, 1987). Cholesterol transport via StAR is the rate-limiting step of corticosteroidogenesis.

Steroidogenic Acute Regulatory (StAR) Protein

StAR is expressed exclusively in steroid-producing tissues. The StAR protein is generated in the cytoplasm and directed to the mitochondrion by an NH₂ (N)-terminal targeting motif. Once localised to the mitochondrion, the targeting motif is cleaved from a 37 kD mature protein to produce a 30 kD transporter. The carboxyl (C)-terminus region of the protein is also crucial to StAR function; truncation of the C-terminus severely decreased the activity of StAR (Arakane *et al.*, 1996). The C-terminus is believed to be crucial for the association of StAR with the outer mitochondrial membrane and conformational changes important to its activity (Yaworsky *et al.*, 2005).

Cholesterol transport by StAR was first observed in rat adrenal cells stimulated with either adrenocorticotrophic hormone (ACTH) or dibutyryl cyclic adenosine monophosphate (cAMP) (Krueger and Ormejohnson, 1983). Cloning and expression of the protein confirmed its role in steroidogenesis (Stocco and Clark, 1996) and StAR-knockout mice have dramatically reduced levels of steroid production and increased levels of stimulatory trophins (owing to a negative feedback mechanism, see section 1.2.5). Moreover, these knockout mice have impaired growth and die prematurely, highlighting the essential functions of corticosteroids (Caron *et al.*, 1997). Patients with mutations in the StAR gene can show an almost complete lack of corticosteroids, and can be lethal in newborn infants. This condition is known as lipoid congenital adrenal hyperplasia (Lin *et al.*, 1995). Although StAR's key role is now established, the mechanism by which it transports cholesterol is not fully understood. However, four modes of action have been proposed (for review see Miller (2007)).

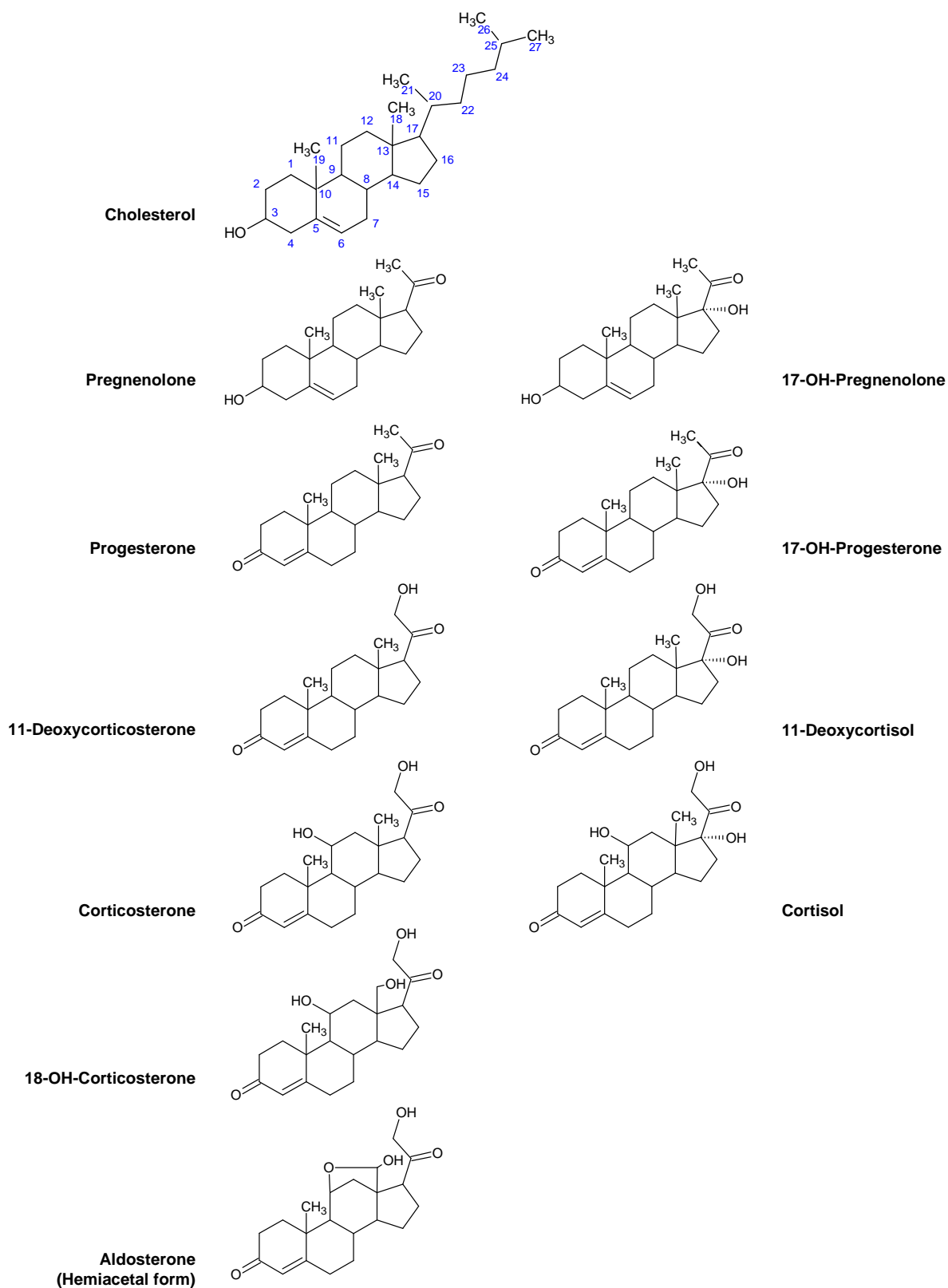


Figure 1-4. Structure of Corticosteroids and Intermediate Compounds.

The carbon atoms are numbered in the cholesterol molecule.

Table 1-3. Identities and Abbreviations of Key Adrenal Steroids.

| Common Name | Abbreviation | Systematic Name |
|--------------------------|--------------|--|
| Cholesterol | C | Cholest-5-en-3 β -ol |
| Pregnenolone | PREG | 3 β -hydroxypregn-5-ene-20-one |
| Progesterone | P | pregn-4-ene-3,20-dione |
| 17-Hydroxypregnenolone | 17-OH-PREG | 3 β ,17 α -dihydroxypregn-5-ene-20-one |
| 17-Hydroxyprogesterone | 17-OH-P | 17 α -hydroxypregn-4-ene-3,20-dione |
| Dehydroxyprogesterone | DHEA | 3 β -hydroxyandrost-5-ene-17-one |
| Androstenedione | A'dione | androst-4-ene-3,17-dione |
| 11-Deoxycortisol | S | 17 α ,21-dihydroxypregn-4-ene-3,20-dione |
| Cortisol | F | 11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione |
| Cortisone | E | 17 α ,21-dihydroxypregn-4-ene-3,11,20-trione |
| 11-Deoxycorticosterone | DOC | 21-dihydroxypregn-4-ene-3,20-dione |
| Corticosterone | B | 11 β ,21-dihydroxypregn-4-ene-3,20-dione |
| 18-Hydroxycorticosterone | 18-OH-B | 11 β ,18,21-trihydroxypregn-4-ene-3,20-dione |
| Aldosterone | Aldo | 11 β ,21-dihydroxypregn-4-ene-3,20-dione-18-al |

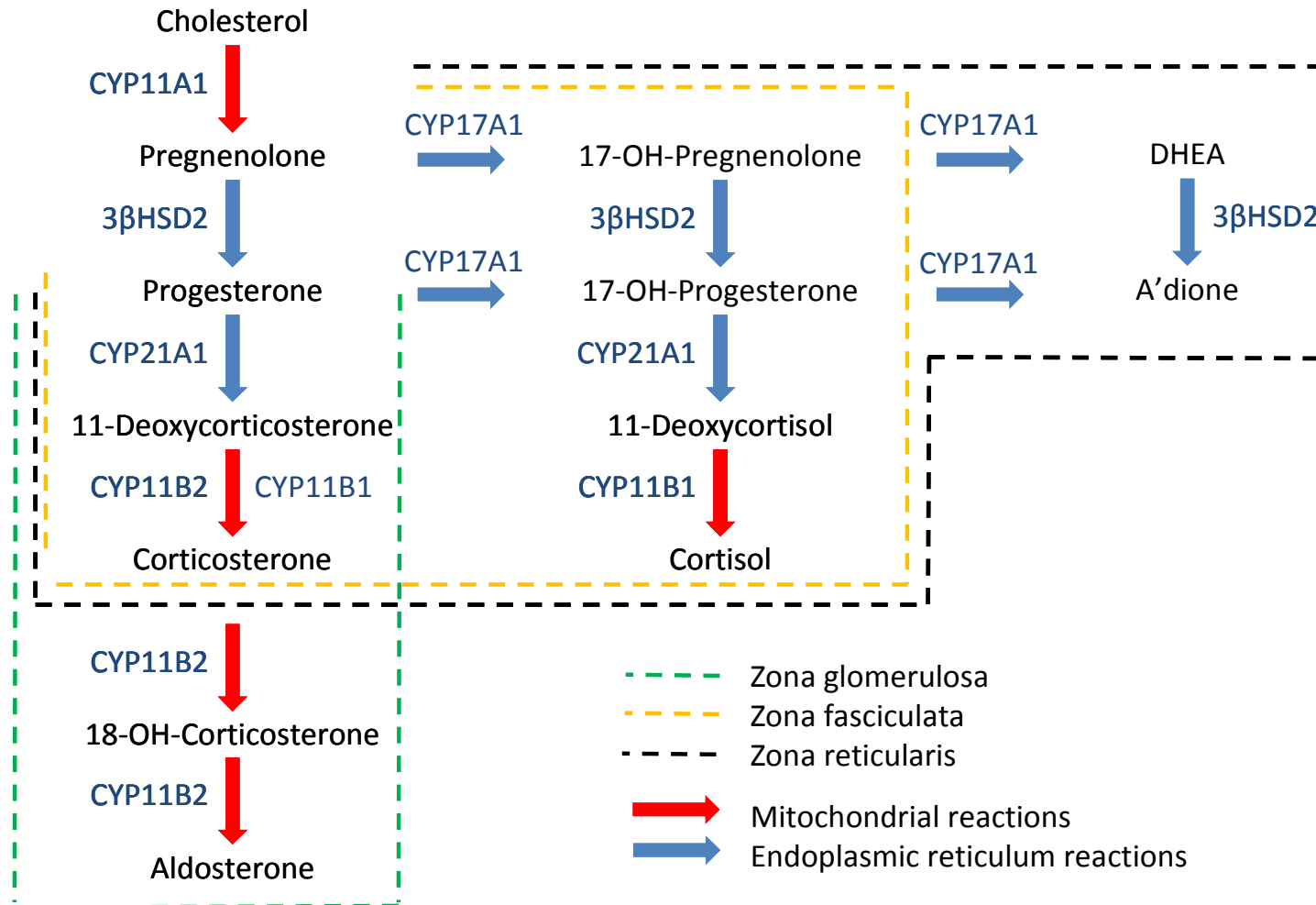


Figure 1-5. Corticosteroid Biosynthetic Pathway.

Pathway of corticosteroid synthesis in the adrenal cortex. Dashed lines represent the zone of the adrenal cortex in which each reaction occurs.

1.2.4 Corticosteroidogenesis

Cholesterol Side-Chain Cleavage (P450scc)

The first step in steroidogenesis involves the cleavage of cholesterol to pregnenolone (Figure 1-5), which is mediated by the cholesterol side-chain cleavage enzyme (CYP11A1 or P450scc). CYP11A1 is encoded by the *CYP11A1* gene, which is located on human chromosome 15. It is expressed in all three zones of the adrenal cortex. The enzyme is located on the inner mitochondrial membrane and catalyses three separate oxidative reactions (Figure 1-4); the first is hydroxylation at C22 and the second the hydroxylation of C20, producing the intermediate compound, 20,22R,hydroxyl-cholesterol. The final reaction catalysed by CYP11A1 is cleavage between C22 and C20, which shortens the side chain of cholesterol by six amino acids, creating the C21 steroid pregnenolone (Figure 1-5). Each reaction requires one molecule of O₂ and one of NADPH, acting via ferredoxin and ferredoxin reductase, to donate electrons. Pregnenolone is the precursor for all steroids of the adrenal cortex (Lieberman and Lin, 2001) and is released into the cytoplasm for further modifications.

3 β -Hydroxysteroid Dehydrogenase (3 β -HSD)

Pregnenolone is converted to progesterone by 3 β -hydroxysteroid dehydrogenase (3 β -HSD). This enzyme is located in the membrane of the smooth endoplasmic reticulum and catalyses three alternative reactions, pregnenolone to progesterone, 17 α -pregnenolone to 17 α -progesterone and dehydroepiandrosterone (DHEA) to androstenedione (Figure 1-5). Two human isoforms of 3 β -HSD have been identified: type I (3 β -HSDI) and type 2 (3 β -HSDII). Each is encoded by a separate gene, both located on human chromosome 1 (Berube *et al.*, 1989; Lachance *et al.*, 1991). The predominant isoform in the human adrenal cortex is 3 β -HSDII. Mutations of this isoform have been identified and associated with congenital adrenal hyperplasia (CAH) (Section 1.4.1.4) (Rheaume *et al.*, 1992).

The corticosteroidogenesis pathway is different in the zona fasciculata and glomerulosa (and reticularis), due to the local enzyme expression profile which is cell type-dependent (Table 1-2). There is little or no evidence of exchange of substrate between zones.

Zona Fasciculata Pathway

The major glucocorticoid, cortisol can be synthesised from pregnenolone or progesterone, but it is probably the pregnenolone pathway that is more important (Figure 1-5). Both compounds result from 17 α -hydroxylase catalysis to produce 17-OH-pregnenolone and 17-OH-progesterone, respectively. As mentioned above 17-OH-progesterone can also be synthesised through the reduction of 17-OH-pregnenolone by 3 β -HSDII.

17 α -Hydroxylase is encoded by the *CYP17A1* gene, located on chromosome 10 and expressed in the zona fasciculata and zona reticularis. It is a dual-functioning enzyme with both 17 α -hydroxylase activity and 17, 20-lyase activity (Figure 1-5). While 17 α -hydroxylation produces 17-OH-pregnenolone and 17-OH-progesterone, 17, 20-Lyase activity (predominant in ZR) removes the residual side chain and is necessary for the synthesis of the C19 androgens; it also converts 17-OH-pregnenolone to DHEA and 17-OH-progesterone to androstenedione.

The next zona fasciculata step involves 21-hydroxylation, catalysed by 21 α -hydroxylase (*CYP21A1*), which converts progesterone and 17-OH-progesterone to 11-deoxycorticosterone (DOC) and 11-deoxycortisol (S), respectively. This enzyme is encoded by the *CYP21A1* gene on human chromosome 6 (White *et al.*, 1986) and expressed in all cell types of the adrenal cortex (Shinzawa *et al.*, 1988).

The final step in cortisol synthesis is the 11-hydroxylation of 11-deoxycortisol, which is catalysed by the 11 β -hydroxylase and occurs in the mitochondria requiring that the 11-deoxy-precursors are again transported to the inner membrane. This enzyme is encoded by the *CYP11B1* gene, which is located on human chromosome 8 (Mornet *et al.*, 1989) and expressed in the zonae fasciculata and reticularis (Ogishima *et al.*, 1992). 11 β -Hydroxylase also converts DOC to corticosterone (B), and also catalyses 18- and 19- hydroxylation, converting DOC to 18-hydroxy-11-deoxycorticosterone (18-OHDOC) and 19-hydroxy-11-deoxycorticosterone (19-OHDOC), respectively. In addition, it converts corticosterone to 18-hydroxycorticosterone (18-OHB). Corticosterone is the principal glucocorticoid in the rat.

Zona Glomerulosa Pathway

The zona glomerulosa is the site of aldosterone synthesis (Figure 1-5). This zone lacks 17 α -hydroxylase and therefore the intermediary substrate synthesised is DOC. To complete aldosterone synthesis, DOC is oxidised in three separate and sequential reactions, each of which is catalysed by a zona glomerulosa-specific enzyme, aldosterone synthase. Aldosterone synthase is encoded by the *CYP11B2* gene, which lies in tandem with the *CYP11B1* gene on human chromosome 8 (Section 1.4.2.1) (Curnow *et al.*, 1991; Kawamoto *et al.*, 1992). Firstly, aldosterone synthase catalyses 11 β -hydroxylation of DOC to produce corticosterone (B), C18 of B is then hydroxylated to produce 18-hydroxycorticosterone (18-OHB). Finally, the hydroxyl group on C18 is hydroxylated, this creates an unstable structure that is resolved by a spontaneous dehydration, producing an aldehyde group at C18 and resulting in the formation of aldosterone (Denner *et al.*, 1995). The reactions catalysed by aldosterone synthase are summarised in Table 1-4 and the structural modification depicted in Figure 1-4.

Table 1-4. Reactions Catalysed by Aldosterone Synthase.

| Substrate | Reaction | Product |
|-------------|---------------------------|-----------------------------------|
| DOC | 11 β -hydroxylation | Corticosterone (B) |
| B | 18-hydroxylation | 18-Hydroxycorticosterone (18-OHB) |
| Aldosterone | '18-oxidation' | Aldosterone |

1.2.5 Control of Secretion of Corticosteroids

Expression of the *CYP11B1* and *CYP11B2* genes is primarily regulated by endocrine trophins, including AngII and ACTH. The extracellular concentration of potassium also contributes to their regulation. Circulating AngII and potassium concentrations are the predominant regulators of aldosterone synthesis and share a common intracellular second messenger pathway that increases transcription of the *CYP11B2* gene and production of aldosterone, whereas ACTH is the key regulator of *CYP11B1* gene transcription and cortisol production. These will be described below. The second level of regulation at the molecular level includes regulation of gene transcription and translational and protein formation. This will be discussed in sections 1.2.6 and 1.2.7.

1.2.5.1 Angiotensin II (AngII)

As previously described, AngII synthesis is under the control of the RAS and its release is stimulated by detection of low blood pressure, sodium depletion or hyperkalaemia. It acts on AT1R, exclusively expressed in the ZG of the adrenal cortex (Section 1.2.1). Antagonising AT1Rs inhibits the AngII dependent increase in *CYP11B2* mRNA in rabbit and rat adrenal glands (Dudley *et al.*, 1990; Hajnoczky *et al.*, 1992). AngII-mediated effects on adrenal function are achieved by activating multiple intracellular second messenger systems, including the scr family of kinases and the 12-lipoxygenase (12-LO) pathway (Bassett *et al.*, 2004). Furthermore, AngII stimulation activates membrane-bound phospholipase C which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Both affect corticosteroidogenesis, IP₃ is soluble and diffuses through the cytoplasm to mobilize bound calcium from endoplasmic reticulum and sacroplasmic reticulum stores, ultimately increasing intracellular free calcium concentrations. By binding calcium-modulating protein (calmodulin or CaM), calcium activates other intracellular pathways, including CaM-dependent protein kinases (CaMK).

Several isoforms of CaMKs are important for corticosteroidogenesis. CaMKI is localized to the ZG and studies using the CAMK inhibitor KN93 and expression vectors in the human adrenocarcinoma cell line, H295R, demonstrated that CaMKI is important for basal *CYP11B2* mRNA expression and also for increased aldosterone production upon stimulation with AngII or K⁺ (Condon *et al.*, 2002). CaMKI is believed to phosphorylate transcription factors that influence *CYP11B2* mRNA transcription (Section 1.2.6).

CaM also activates the CaM-dependent protein phosphatase, calcineurin. A recent study showed that calcineurin mRNA is increased from basal in H295R cells stimulated with AngII, and manipulating calcineurin levels by pharmacological agents, short interfering RNAs (siRNAs) or adenovirus transfer suggest it is a new calcium-dependent regulator of *CYP11B2*, at least *in vitro* (Yamashiro *et al.*, 2010).

In contrast to IP₃, DAG remains at the cell membrane, initiating translocation of protein kinase C (PKC) to the plasma membrane, then its activation. Several

members of the PKC family have been implicated in adrenal steroidogenesis (Lehoux *et al.*, 2001) and many intermediates, including arachidonate and 12-HETE, may be involved in the stimulation of aldosterone secretion. PKC does not appear to have direct effects on *CYP11B2* transcription but may act by inhibiting the *CYP17A1* gene, thus making aldosterone synthesis the preferred route of steroidogenesis (Bassett *et al.*, 2004).

While AngII is a potent stimulator of *CYP11B2* expression, it also influences other genes that may contribute indirectly to increased production of aldosterone. A recent gene microarray study of stimulated H295R cells identified several genes which has altered expression compared to non-stimulated cells. For example, members of the nerve growth factor 1B family successfully increased aldosterone production when exogenously expressed in cells (Romero *et al.*, 2007).

1.2.5.2 Potassium Ions (K^+)

Potassium ions (K^+) stimulate aldosterone production by both RAS-dependent (alterations in electrolyte balance can modulate the production of AngII synthesis) and RAS-independent direct effects on the adrenal cortex. Increased extracellular K^+ concentration ($[K^+]_e$) increases aldosterone synthesis and secretion.

Increased $[K^+]_e$ causes the ZG cell membrane to depolarise, opening cell membrane voltage-gated calcium channels. This occurs in a concentration dependent manner; changes within physiological ranges activate T-type calcium channels, whereas higher concentrations activate L-Type calcium channels (Lotshaw, 2001). Calcium flux into the cells rapidly increases the intracellular free calcium concentration which, in turn, activates calmodulin and CaM kinases to stimulate aldosterone production in a similar manner to AngII (Section 1.2.5.1) (Pezzi *et al.*, 1997).

Adrenal glomerulosa cells are highly sensitive potassium-sensor cells and exhibit a rapid response to very small changes in $[K^+]_e$; for example, a small potassium chloride infusion in normal volunteers, which did not significantly change plasma $[K^+]$, caused an increase in plasma aldosterone within 30 minutes (Himathongkam *et al.*, 1975). Moreover, in an *in vitro* system that tends to be

less sensitive to stimulation, an increase of only 1 mM of K^+ was sufficient to double the production of aldosterone in primary rat glomerulosa cells (Pralong *et al.*, 1992). This sensitivity arises due to a very large negative resting membrane potential of the ZG cells, consequentially the membrane is highly permeable to potassium. The identity of the channel responsible for maintaining the membrane at this voltage was identified in 2000. The expression of TWIK-related acid-sensitive K^+ (TASK) channel was verified in ZG cells using single-cell PCR. *Xenopus Laevis* oocytes were used as a model to test the function of these channels and their susceptibility to inhibitors (Czirjak *et al.*, 2000). The voltage-insensitive nature of this class of channels allows them to be constitutively open in ZG cells. Reduction of the levels of TASK mRNA by short interfering RNA (Section 1.5.1) directed against the TASK channel transcript attenuated the potassium current. Further studies identified TASK-3 (KCNK9) as the predominant sub-type in ZG cells (Czirjak and Enyedi, 2002). Homozygous knockout mice have confirmed a role for TASK channels in the regulation of aldosterone production and interestingly show that TASK knockout mice have impaired adrenal zonation, adrenal cortex function and developed a phenotype similar to primary aldosteronism (Section 1.4.1) (Heitzmann *et al.*, 2008). Taken together, these studies demonstrate outward rectifying K^+ channels are required for corticosteroid synthesis and for the correct development of the adrenal gland.

1.2.5.3 Adrenocorticotrophic Hormone (ACTH)

Adrenocorticotrophic hormone (ACTH) is the principal secretagogue of cortisol production but also exerts a modest stimulatory effect on aldosterone secretion which is dependent on sodium status. ACTH is the main active component of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1-6). Typically, central neural pathways are activated by stimuli such as stress and initiate the release of corticotrophin-releasing hormone (CRH) by the hypothalamus. CRH is transferred along the hypophyseal portal circulatory system to stimulate the release of pro-opiomelanocortin (POMC), from which ACTH is excised by prohormone convertase in the anterior pituitary gland. Cleavage releases ACTH and several other biological peptides, including, melanocyte stimulating hormone and β -endorphin. ACTH secretion exhibits a diurnal rhythm, highest in early morning and lowest late at night.

ACTH is released from the anterior pituitary gland into the systemic circulation and binds with high affinity to the ACTH-receptor. The ACTH-receptor is expressed in adrenal cortical cell membranes and is a member of the G-protein-coupled receptor family. ACTH binding activates adenylate cyclase increasing the intracellular level of cyclic adenosine monophosphate (cAMP) which activates protein kinase A (PKA). These second messengers stimulate *CYP11B1* gene transcription via transcription factors (Section 1.2.6).

ACTH secretion is essential to correct adrenal formation, growth and corticosteroid production whereas, hyper-secretion of cortisol leads to hypertension and Cushing's Syndrome (Section 1.4). To maintain cortisol homeostasis ACTH secretion is subject to negative feedback regulation (Figure 1-6). Cortisol prevents further ACTH secretion by binding to glucocorticoid receptors (GR) in the hypothalamus.

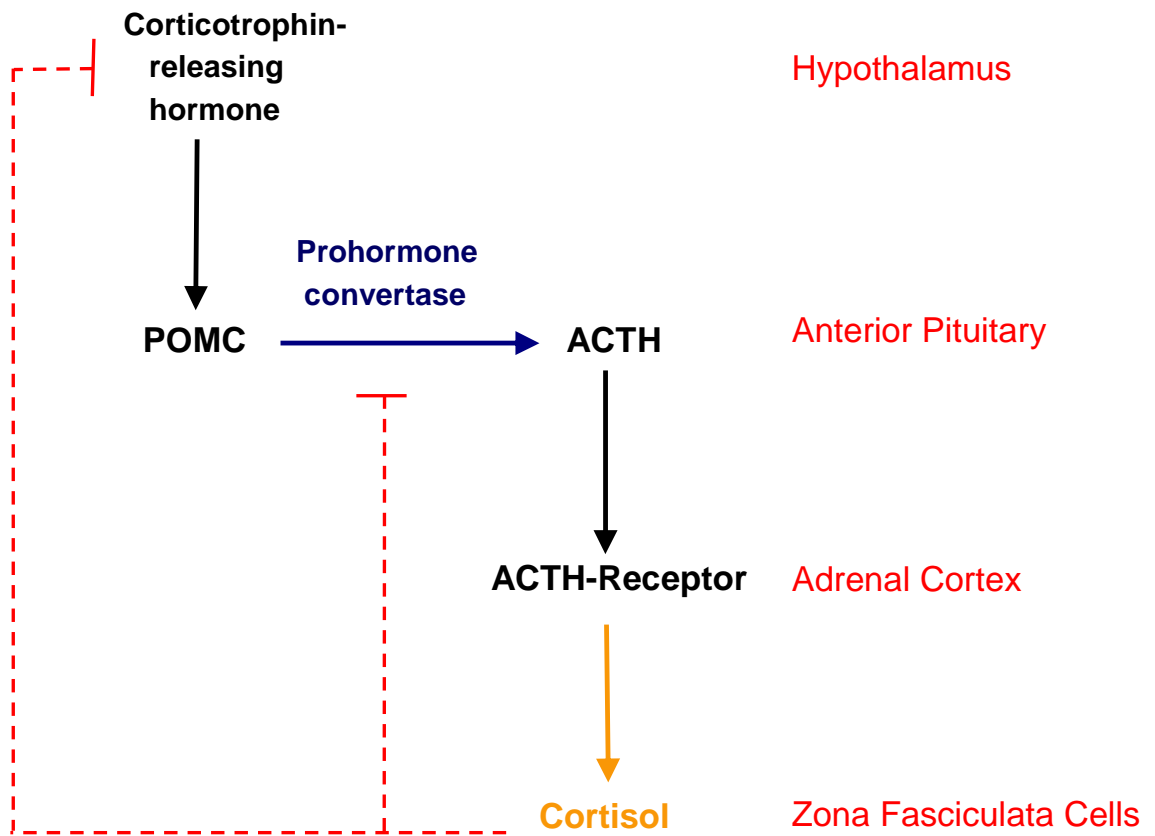


Figure 1-6. Hypothalamic Pituitary Axis Regulation of Cortisol Secretion.

POMC: pro-opiomelanocrtin; ACTH: adrenocorticotrophic hormone.

1.2.6 Transcriptional Regulation of *CYP11B* Genes

Transcription factors (TF) are the largest class of DNA-binding proteins. TFs bind to a specific recognition site (or sites) in the 5'-UTR/ promoter region of a gene and function to regulate gene transcription. There are numerous TFs which are cell- and pathway-dependent. Their expression is tightly regulated and they often work in combination with co-factor proteins. They form one of the most important regulatory control systems of gene transcription and understanding how they function with regard to a particular gene can give an important insight in to normo- and patho-physiology. Variation in the 5' promoter structures of the *CYP11B1* and *CYP11B2* genes affect blood pressure regulation and hypertension; this may be because altered sequences influence TF binding this will be discussed further in section 1.4.2.2.

Prior to characterisation of the human *CYP11B1* and *CYP11B2* promoters or of the equivalent regions in rats or mice, the single *CYP11B* bovine gene had been investigated. Several conserved *cis*-elements, known as Ad1 - Ad6 (Adrenal 1-6) were identified (Figure 1-7) (Kirita *et al.*, 1990). Each DNA-binding motif was predicted to bind a nuclear receptor (Bassett *et al.*, 2004). These have since been investigated in the human genes and the findings of these, together with other 5' regulatory mechanisms, will be discussed below.

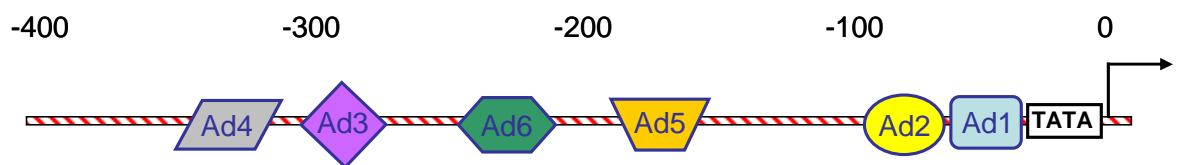


Figure 1-7. Regions of Transcriptional Importance in the *CYP11B* Genes.

Ad1-6: adrenal 1-6. Not to scale. (Kirita *et al.*, 1990; Bassett *et al.*, 2004)

1.2.6.1 Transcription Regulation of *CYP11B2*

In addition to the *cis*-elements detailed above, studies using reporter constructs containing the *CYP11B2* promoter, from which had series of sequence deletions had been made, identified two additional regions of transcriptional importance: a cAMP response element (CRE) and a NBRE-1 site (neuronal growth factor-induced clone B (NGRI-B) response element (Figure 1-8). This study also

confirmed the importance of the Ad5 site in human *CYP11B2* transcriptional regulation (Clyne *et al.*, 1997).

The CRE site binds several TFs, including activating transcription factors 1 and 2 (ATF1 and ATF2) and CRE-binding protein (CREB) (Bassett *et al.*, 2004). All of these TFs can interact with the activation protein-1 (AP-1) complex, specifically the *JUN* and *FOS* genes. Moreover, AngII and ACTH both increase levels of c-fos (the protein product of *FOS*), which can dimerise with Jun to form the AP-1 transcription complex (Romero *et al.*, 2007).

The NBRE-1 site binds members of the NGFI-B nuclear orphan receptor superfamily. Members of the NGFI-B family include NGFI-B, NR4A2 (NURR1), and NR4A3 (NOR1). The family is a member of the *Nur* nuclear receptor family of TFs. They are highly expressed in the adrenal cortex, in a zone-specific manner. Two members of the family, NR4A1 and NR4A2 (nuclear receptor subfamily 4 group A member 1 and 2), are targets for AngII stimulation; NR4A1 also responds to ACTH and NR4A2 to changes in K⁺ concentration. Both have been shown to regulate expression of three steroidogenic-related genes: *3β-HSDII*, *CYP11A1* and *CYP11B2* (Bassett *et al.*, 2004; Nogueira *et al.*, 2009). A recent study conducted in H295R cells investigated the NBRE-1 site and identified a stimulatory transcriptional role for the other NGFI-B family members, NURR1 and ATF/CREB, a synergistic relationship between them was observed (Nogueira and Rainey, 2010).

The Ad4 site at -344 to -366 of the *CYP11B2* promoter is a consensus match for the steroidogenic factor-1 (SF-1) TF binding site (Figure 1-8). SF-1 is a monomeric orphan nuclear receptor required for correct development and function of the adrenal gland. The site has been the focus of a great deal of research because of the common polymorphism at this site observed in certain hypertensive population; this will be discussed later, in section 1.4.2.2. This research has revealed a positive regulatory function for other genes in the corticosteroid pathway including all steroid hydroxylase genes (Bassett *et al.*, 2002). However, deletion of this sequence (Ad-4) (Section 1.2.6) was without effect on *CYP11B2* expression and further analysis suggested an inhibitory role for SF-1 on *CYP11B2* (Bassett *et al.*, 2002).

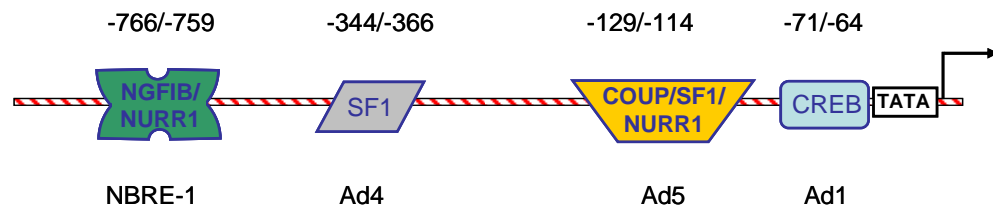


Figure 1-8. Transcription Factor Binding Sites on the *CYP11B2* Promoter.

Not to scale. Adapted from Bassett *et al.*, (2004).

1.2.6.2 Transcriptional Regulation of *CYP11B1*

Reporter construct analysis of the human *CYP11B1* promoter sequence suggested that, under basal and ACTH stimulation, there are two important regulatory regions: Ad-1, a cAMP response element (CRE), and Ad-4, a SF-1 consensus sequence (Figure 1-9) (Wang *et al.*, 2000). The CRE site is nearly identical to that observed in the *CYP11B2* gene and mutational studies have proved that it is essential for *CYP11B1* expression during basal conditions (Bassett *et al.*, 2000).

The *CYP11B1* Ad-4 site differs from the *CYP11B2* site by only one nucleotide, but this is sufficient to make SF-1 an effective regulator of *CYP11B1* transcription but not of *CYP11B2* (Bassett *et al.*, 2002). Moreover, *CYP11B1* mRNA is more abundant in H295R cells that over-express SF-1 compared to unmodified cells, and this increase is greater when cells are stimulated with AngII. Targeting SF-1 with an siRNA prevents these increases in *CYP11B1* mRNA (Ye *et al.*, 2009), giving more evidence of the differential effect of SF-1 on the *CYP11B1* and *CYP11B2* genes, which helps to determine the pattern from steroid output of the adrenal cortex. Studies from the same group also identified a regulatory role for SF-1 on several other corticosteroidogenic enzymes, including StAR, CYP11A1 and 17 α -hydroxylase (Bassett *et al.*, 2002).

Investigations of the transcriptional regulation of the *CYP11B1* and *CYP11B2* genes have identified numerous transcriptional active proteins, many of which are regulated by AngII, K⁺ and ACTH. However, this area of regulation requires further research and, to date, no good evidence exists to support a role for changes in transcriptional regulation (via TFs) and the development of essential hypertension.

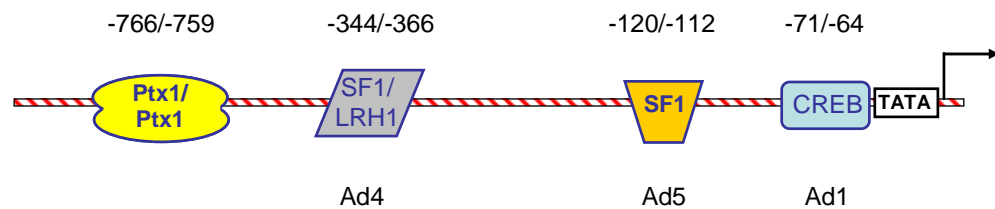


Figure 1-9. Transcription Factor Binding Sites on the *CYP11B1* Promoter.

Not to scale. Adapted from Wang *et al.*, (2000).

1.2.7 Post-Translational Regulation

Another level at which gene expression can be regulated is by post-translational modification. This includes acetylation, phosphorylation and ubiquitination. There is no reported evidence of post-transcriptional regulation of the *CYP11B1* and *CYP11B2* genes and it is rarely observed in other CYP450 enzymes. However, there is one example: CYP17A1 can be phosphorylated at serine and threonine residues and this can alter the function of the enzyme, with phosphorylation favouring 17,20-lyase activity (Section 1.2.4) (Zhang *et al.*, 1995). Another post-translational modification that is relevant to corticosteroids production is the phosphorylation of bovine adrendoxin (Bureik *et al.*, 2005) and modification of TFs involved in regulating the *CYP11B1* and *CYP11B2* genes (Camarota *et al.*, 2001;Nogueira and Rainey, 2010).

To summarise, the understanding of transcriptional regulation of the *CYP11B1* and *CYP11B2* genes is relatively well studied. Until recently, it was believed that transcriptional regulation was the dominant factor regulating protein synthesis but novel regulatory mechanisms and regulatory molecules for example microRNAs, has opened new avenues of investigation. microRNAs will be introduced in section 1.5.2.

1.3 Physiological Actions of Corticosteroids

Both aldosterone and cortisol exert their effects by binding to specific nuclear receptors. Nuclear receptors belong to a large superfamily with 49 human members that can be sub-divided on the basis of their receptor dimerization and DNA-binding characteristics. Class I comprises the estrogen receptors (ER α and ER β), the progesterone receptor (PR), the androgen receptor (AR), the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These receptors all share similar structural characteristics; with GR and MR being the most closely related phylogenetically (Mangelsdorf *et al.*, 1995). All have an N-terminal domain, a DNA-binding domain and a C-terminal ligand-binding domain (Arriza *et al.*, 1987).

Unlike other receptors which span or are associated with the plasma membrane, steroid receptors are located in the cellular cytosol (Figure 1-10). In their inactive form, steroid receptors are associated with a complex of chaperone proteins, including heat shock proteins (Hsp90 and Hsp50) (Funder, 1997). Upon binding an agonist, the receptor undergoes a conformational change, dissociates from the chaperone proteins, dimerises and is translocated to the nucleus. The steroid receptor dimer functions as a TF in common with all ligand-bound steroid-binding receptors. The steroid receptor dimer binds consensus hormone-response elements (HRE), which are approximately 15 nucleotides in length and are located in 5' enhancer or promoter regions of target genes (Figure 1-10). Two zinc fingers present in the DNA-binding domain of the MR are responsible for tethering it to the HRE. Gene transcription is initiated, leading to the synthesis of new protein.

1.3.1 Mineralocorticoids

The MR is encoded by the *NR3C2* gene, located on human chromosome 4 (Morrison *et al.*, 1990) and is mainly expressed in polarised epithelial cells in such organs as the kidney, colon and salivary glands. It play a key part in regulating electrolyte metabolism and acid base balance and more recently, it has been identified in non-epithelial cells in the brain, heart and vascular tissue (Funder, 2005).

The MR has poor specificity with aldosterone and cortisol demonstrating approximately equal binding affinity. The circulating plasma levels of aldosterone are relatively low, (<1nmol/L) and half is bound to albumin, and the rest readily available for receptor binding. In contrast, the circulating levels of cortisol are 100- to 1000-fold higher and only 5% is bound. Thus, it would be expected that cortisol would be the dominant ligand of MR. However, this is not the case because MR is protected from cortisol by 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSDII) which is co-localised with MR in epithelial cells (Figure 1-10) (Funder *et al.*, 1988; Edwards *et al.*, 1988). 11 β HSDII metabolises cortisol to cortisone, which does not bind the MR. As previously mentioned, DOC has mineralocorticoid properties and may have a higher affinity for MR than its physiological potency implies. A recent study demonstrated that MR, in epithelial tissue, also co-localises with a member of the aldo-keto-reductase family, AKR1C3. The investigators found that AKR1C3 can protect MR from inappropriate DOC activation by converting it to 20 α -hydroxy-DOC, an inactive steroid (Sharma *et al.*, 2006).

In epithelial tissues, the principal function of aldosterone-bound MR is to increase sodium reabsorption, linked to fluid retention, and increase excretion of potassium and hydrogen ions. Within the kidney, MR is expressed in the distal convoluted tubule and the cortical collecting duct (CCD) (Todd-Turla *et al.*, 1993). The main cell type in this region, responsible for salt homeostasis are principal cells, which lie adjacent to intercalated cells.

The primary function of intercalated cells is in the control of acid-base balance. They express H⁺-ATPase channels and MRs which are both regulated by aldosterone. In addition to MR, several channels and transporters (discussed below) are expressed within principal cells to facilitate the movement of Na⁺ and K⁺ across apical and basolateral membranes, and these are all subject to aldosterone regulation.

The amiloride-sensitive epithelial sodium channel (ENaC) is located on the apical membrane and allows Na⁺ to move from the lumen of the nephron down an electrochemical gradient into the principal cell. This is the rate-limiting step of Na⁺ reabsorption (Horisberger, 1998). Also expressed on the apical membrane is the renal outer medullary potassium channel (ROMK), which facilitates passive

movement of K^+ across the membrane. The basolateral membrane contains three transporters, the Na^+/K^+ ATPase, the Na^+/H^+ exchanger (NHE) and the ATP sensitive K^+ channel (Figure 1-10). These all function to complete Na^+ reabsorption into the blood while maintaining the electrochemical gradient for transport across the apical membrane.

There are 8 human isoforms of NHE and each has different expression patterns and regulatory mechanisms. NHE3 has been identified as the isoform most sensitive to aldosterone and its effect is blocked by spironolactone, an MR antagonist (Drumm *et al.*, 2006). It is believed that aldosterone works in conjunction with the epidermal growth factor receptor to activate NHE3 expression and increase trafficking of the transporter to the membrane surface (Thomas and Harvey, 2010). Aldosterone also has slower effects which may be mediated by protein kinase C (PKC) or by the aldosterone-inducible proteins serum- and glucocorticoid-regulated kinase 1 (SGK1) and channel-inducing factor (CHIF) (Section 1.3.1.1) (Odermatt and Atanasov, 2009; Thomas and Harvey, 2010).

Finally, aldosterone regulation of the ENaC is essential for salt and fluid homeostasis and the steroid can alter the rate of reabsorption in two ways: by altering the number and density of ENaC channels on the cells' surface or by changing the opening probability of ENaC. The former is likely to be a chronic (3-24 hours) effect requiring synthesis of new protein, whilst the latter is more likely to be an acute effect (<3 hours). ENaC is composed of three subunits (α , β and γ) and is continually recycled from the membrane surface and intracellular pools (Butterworth *et al.*, 2005). In rats treated with either aldosterone or dexamethasone, a synthetic corticosteroid, ENaC subunit mRNA was increased in a cell-type specific manner; in the cortex of the kidney, there was an increase in the α -ENaC mRNA, whereas in the distal colon both β -ENaC and γ -ENaC subunits were increased (Asher *et al.*, 1996). A proposed mechanism for direct aldosterone action is that ligand-bound MR binds to the 5' regulatory regions on the *ENaC* gene increases transcription (Mick *et al.*, 2001). Additionally, aldosterone can have indirect effects on ENaC, which are mainly mediated through Aldosterone-induced Proteins (AIP) (Section 1.3.1.1).

1.3.1.1 Aldosterone-Induced Proteins

The most widely studied AIP is SGK1, a serine-threonine kinase upregulated in response to mineralocorticoids (Figure 1-10). Experiments conducted in epithelial cell lines, adrenalectomised mice and using ectopic expression of SGK1 in *Xenopus Laevis* oocytes identified a regulatory role for SGK1 through increased ENaC activity (Chen *et al.*, 1999). Moreover, SGK1 mRNA was shown to increase in response to dexamethasone stimulation (Chen *et al.*, 1999). Further evidence of SGK1 involvement in salt homeostasis came from homozygous *sgk1* knockout mice, mice on a low NaCl diet had impaired salt reabsorption and low blood pressure, despite high circulating aldosterone levels (Wulff *et al.*, 2002). SGK1 action can affect sodium transport; for example, it phosphorylates the ubiquitin protein ligase, neural precursor cell expressed, developmentally down-regulated 4-2 (Nedd4-2) (Figure 1-10). In its unphosphorylated state Nedd4-2 ubiquitinates ENaC, which directs the channel for degradation but phosphorylation inactivates Nedd4-2 and prevents this, leading to increased ENaC at the apical membrane (Debonneville *et al.*, 2001).

Another AIP is the corticosteroid hormone induced factor or channel-inducing factor (CHIF), a small membrane protein (FXFD family) associated with the Na⁺/K⁺ ATPase transporter in renal tissue (Figure 1-10). CHIF mRNA expression is increased in rats administered aldosterone (Brennan and Fuller, 1999). CHIF is homologous to the γ -subunit of Na⁺/K⁺ ATPase and it is thought that it increases Na⁺/K⁺ ATPase affinity for Na⁺, thus enhancing the reabsorption rate of Na⁺. Increased transport of Na⁺ increases the electrochemical gradient of Na⁺, thus also increasing transport across the apical membrane (Beguín *et al.*, 2001).

The mRNA of endothelin-1, another AIP (Figure 1-10), is rapidly increased in response to aldosterone in a rat smooth muscle cell lines (Wolf *et al.*, 2006). This increase was also observed in adrenalectomized rat kidneys and colon following aldosterone treatment (Wong *et al.*, 2007). More recently, aldosterone-induced endothelin-1 was shown to be specifically expressed in the renal collecting duct and its induction to be mediated by two putative HRE present in the endothelin-1 promoter (Stow *et al.*, 2009). The mechanisms of endothelin-1 actions are unclear but *in vitro* and *in vivo* experiments have

demonstrated that it can increase *SGK1* mRNA and may exert its actions via this AIP (Wolf *et al.*, 2006).

The importance of other AIPs is less clear. GTP-dependent signalling protein K-ras2 (KiRasA) increases ENaC opening time *in vitro* but experiments in rats detected increased expression only in the colon and not in the kidney (Odermatt and Atanasov, 2009). Another AIP with an unclear mechanism or relevance is glucocorticoid-induced leucine-zipper (GILZ) protein. This antagonises extracellular signal-regulated kinase (ERK), a negative regulator of ENaC; thus GILZ increases Na⁺ reabsorption (Bhalla *et al.*, 2006).

Taken together, there is substantial evidence that aldosterone affects several transporter systems in the nephron, both directly and indirectly, via AIPs, and that this facilitates the homeostatic regulation of sodium and potassium transport and water balance, which are key factors in blood pressure control.

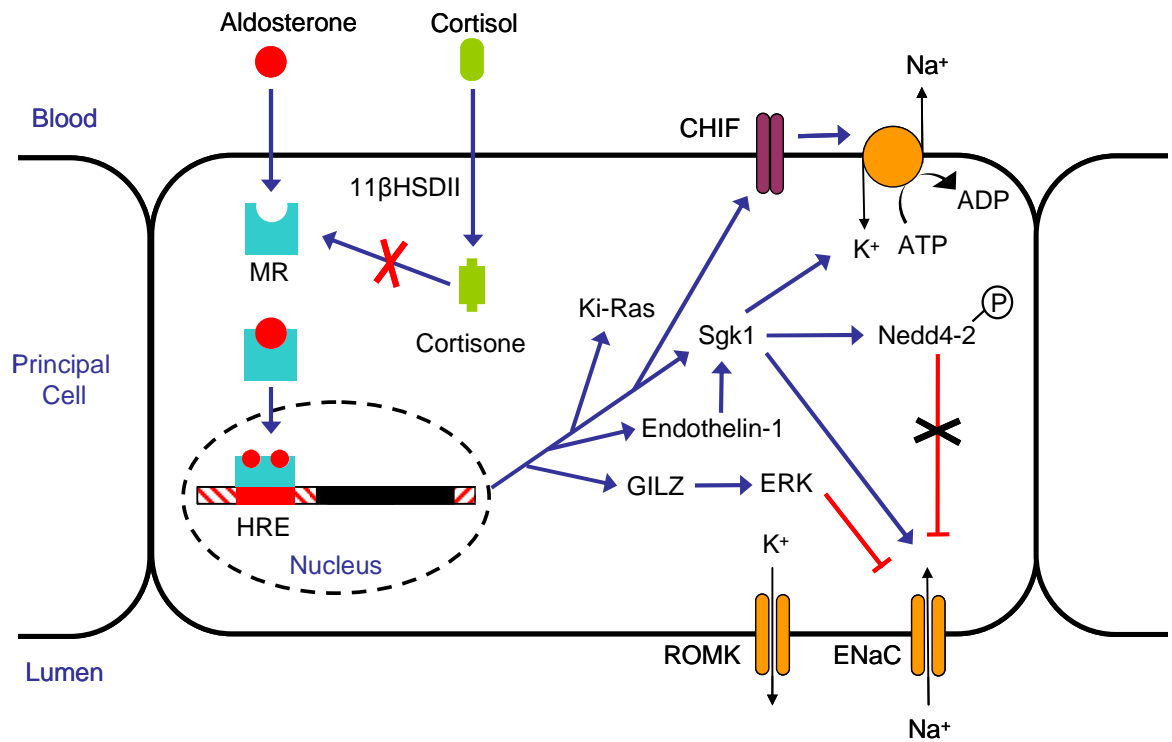


Figure 1-10. Classical Mechanism of Aldosterone Action in Epithelial Cells.

Aldosterone binds to the mineralocorticoid receptor (MR). This complex dimerizes and translocates to the nucleus. The dimer binds to a hormone-response element (HRE) on the gene promoter and influences transcription. Cortisol is inactivated by 11 β -dehydrogenase II (11 β HSDII) to form cortisone. ENaC: epithelial sodium channel; GILZ: glucocorticoid-induced leucine zipper protein; ERK: extracellular signal-regulated kinase; ROMK: renal outer medullary potassium channel; Sgk1: serum- and glucocorticoid-regulated kinase 1; CHIF: corticosteroid hormone induced factor; Nedd4-2 neural precursor cell expressed, developmentally down-regulated 4-2.

1.3.1.2 Non-Genomic Actions of Aldosterone

In addition to acting as a ligand-dependent TF in combination with MR, aldosterone has rapid cellular effects which cannot be explained by changes in gene regulation. Classical aldosterone actions mediated via the MR are subject to a lag phase (1-2 hours), can be blocked by MR inhibitors and are susceptible to transcription or translational blockade, for example with puromycin or actinomycin D (Edelman *et al.*, 1963; Horisberger and Diezi, 1984). However, physiological responses to aldosterone have been observed over shorter time frames (1-2 minutes) and are not affected by blockade of the nuclear MR (Funder, 2005).

Aldosterone's capacity to increase sodium exchange in canine erythrocytes was observed in the 1960s, a response which obviously does not involve the nucleus (Spach and Streeten, 1964). This was followed by elegant studies in human leucocytes and the idea of non-genomic aldosterone action was founded (Wehling *et al.*, 1989). A striking finding of these studies was that membrane binding sites had very low K_d , indicating high affinity. These mechanisms have since been demonstrated in renal epithelial cells (Gekle *et al.*, 1996), in several primary renal cell cultures (Koppel *et al.*, 2003), in vascular smooth muscle cells (Christ *et al.*, 1995) and in primary human colon cells (Doolan *et al.*, 1998). *In vivo* non-genomic effects of aldosterone have been supported by MR knockout mice; *ex vivo* skin cells from MR^{-/-} mice respond rapidly (<2 minutes) to aldosterone stimulation (Haseroth *et al.*, 1999). Several reports show rapid vasoconstriction in response to aldosterone however, recent evidence demonstrates vasodilation, that may be mediated via nitric oxide; the physiological significance of this mechanism of action in light of the classical mechanism remains unclear (Grossmann and Gekle, 2009).

It has been postulated that the rapid response may be attributed to a novel receptor, the MR receptor working in a novel, non-genomic way or that classical gene transcription may facilitate a signalling cascade more rapidly than is currently estimated (Vinson and Coghlan, 2010). Evidence suggests that this rapid response mechanism involves changes in intracellular free Ca^{2+} concentration, as well as several second messenger systems (Grossmann and Gekle, 2009). A list of proteins induced by aldosterone has been derived from

2D-electrophoresis experiments of yeast extracts, which do not express nuclear receptors (Bohmer *et al.*, 2006). This is promising work but functional experiments will be required to understand fully the mechanism involved.

1.3.1.3 Aldosterone Actions in Non-Epithelial Cells

MR expression in non-epithelial tissues has revealed a novel mechanism of aldosterone action in tissues other than epithelia. MR expression has been identified in many cell types including vascular cells (smooth vascular and endothelial), cardiac cells (fibroblast and cardiomyocytes), neuronal cells (particularly in the CNS) and circulatory cells (monocytes, leucocytes and lymphocytes) (Viengchareun *et al.*, 2007; Odermatt and Atanasov, 2009). With the exception of the vascular cells, the MR is not co-expressed with 11βHSDII. Thus, activation of MR will be dominated by glucocorticoids in these tissues (Alzamora *et al.*, 2000).

The clinical significance of aldosterone's blood pressure controlling properties will be discussed later in section 1.4, but it is worth noting here that aldosterone's actions in the cardiovascular system extend beyond blood pressure control and are often detrimental. An early study showed that rats on a high salt diet had greater cardiac collagen accumulation, fibrosis and left ventricular hypertrophy when given aldosterone in comparison to control animals (Brilla and Weber, 1992). This phenomenon is called cardiac remodelling. A subsequent study showed that these effects were dependent on high salt status and independent of blood pressure changes (Young *et al.*, 1995). Furthermore, a recent study conditionally over-expressing MR in endothelial cells of mice found the animals to have increased vascular resistance, vasoconstriction and hypertension (Cat *et al.*, 2010).

The mechanism of aldosterone-induced cardiovascular damage is unclear; a number of genes which appear to be regulated by MR have been identified, including those involved in collagen synthesis, inflammation and cell proliferation (Viengchareun *et al.*, 2007). Prior to these observations, cardiac remodelling had been attributed to indirect effects of aldosterone and DOC mineralocorticoid activity principally raised blood pressure. However, an important clinical heart failure trial (RALES) in the late 1990s showed that the

addition of spironolactone (an MR antagonist) to standard treatment reduced the risk of morbidity and mortality when compared to standard treatment alone (Pitt *et al.*, 1999).

1.3.2 Glucocorticoids

The majority of cortisol's effects are mediated through binding to the glucocorticoid receptor (GR). GR activation is identical to that of MR; the active GR dimer complex translocates to the nucleus and binds to specific glucocorticoid-responsive elements (GRE) located in promoter regions of target genes stimulating their expression. This process is known as transactivation. An alternative method of GR function is transrepression, involving negative GRE (nGRE) sites. Once bound, these act to repress gene transcription. An example of transrepression is the negative feedback mediated by nGRE located in the pro-opiomelanocortin (*POMC*) gene, which is an ACTH precursor gene (Section 1.2.5.3) (Drouin *et al.*, 1993).

The GR is ubiquitously expressed (Ballard *et al.*, 1974) and thus cortisol has a wide range of target tissues and can facilitate a range of effects (Sapolsky *et al.*, 2000). The primary role of cortisol is to mediate the stress response and metabolism of fat and carbohydrate, leading to increased glycogenolysis and blood glucose levels in competition with insulin. Other known roles of cortisol include suppression of the inflammatory and immune responses and, most relevant to this review, regulation of blood pressure. The mechanisms by which cortisol can regulate blood pressure are complex and several examples are listed in Table 1-5.

Table 1-5. Cardiovascular Effects of Glucocorticoids (Walker, 2007).

| Target Cell Type | Via GR | Via MR |
|-----------------------------|---|---|
| Vascular Smooth Muscle Cell | Increased contractility Decreased proliferation Decreased migration | Increase perivascular inflammation Changes in vasotone |
| Endothelial Cell | Decreased vasodilation Decreased angiogenesis | Changes in vasotone |
| Myocardium | | Increased fibrosis |
| Macrophage | Changes in cytokines Increase apoptosis | |
| Non-Cardiovascular Organs | Obesity Hypertension Dyslipidaemia Insulin resistance Glucose intolerance Prethombotic | Hypertension Prothrombotic |

1.4 The Role of Corticosteroids in Hypertension

The previous sections have discussed the synthesis, regulation and function of corticosteroids and their role in blood pressure homeostasis. The detrimental effects of hypertension and its role as a risk factor for cardiovascular disease was described in section 1.1.2. In approximately 5% of hypertensive patients, there is a definable cause of hypertension, known as secondary hypertension. The following sections describe these rare but important causes. They show clearly that corticosteroids can cause hypertension. More recent studies indicate that corticosteroid metabolism may also be altered in the remaining 95%, so-called essential hypertension. This will be reviewed with particular attention to the *CYP11B1* and *CYP11B2* genes.

1.4.1 Secondary Hypertension

For the purpose of this thesis, and to highlight the importance of the adrenal cortex and hypertension two types of secondary hypertension, first primary aldosteronism which will also introduce the aldosterone to renin ratio and its use as a diagnostic tool; secondly monogenic forms of hypertension will be introduced, paying particular attention to those in which mineralocorticoid production is altered.

1.4.1.1 Primary Aldosteronism

Primary aldosteronism (PA) (or primary hyperaldosteronism) is an important cause of secondary hypertension characterised by increased aldosterone concentration and associated with normal renin levels (Section 1.4.1.2). PA was first recognised by Dr Jerome W. Conn in 1954 in a 34-year old woman with hypertension and muscle spasms and weakness due to hypokalaemia. This led to a diagnosis of excess production of the salt-retaining corticoid from the adrenal cortex. Surgical intervention revealed a right-sided adrenal tumour which, when removed, left the patient free of all symptoms (Conn, 1955). In the following ten years, Conn identified 145 similar cases and proposed that a proportion of essential hypertension cases might have primary aldosteronism.

Since then, the number of essential hypertensive patients with primary aldosteronism is estimated to be 10% of patients (Funder *et al.*, 2008) although in accepting this figure, it is important to stress that the definition of the disorder has been modified since Conn's original description.

Currently, there are several sub-classifications of primary aldosteronism which differ in terms of pathology and treatment. The subtypes of primary aldosteronism are listed in Table 1-6. One of the main causes is a small, solitary aldosterone-producing adenoma (APA), similar to the first case described by Conn; currently the aetiology of APA is unknown. Adenomas are autonomous sources of aldosterone synthesis that is not under the control of AngII stimulation. In cases of the main type of PA, bilateral idiopathic hyperplasia (IHA) the ZG is hyper-responsive to AngII, which leads to the expansion of the ZG. Patients with APA have higher blood pressures than those with IHA (Blumenfeld *et al.*, 1994). Diagnosis and treatment of PA is important as they have a higher rate of cardiac events (stroke, myocardial infarction *etc*) than age-, gender- and blood pressure-matched essential hypertensive patients (Milliez *et al.*, 2005).

Table 1-6. Subtypes of Primary Aldosteronism (taken from Young (2007)).

| Pathology | Prevalence |
|---|------------|
| Aldosterone-producing adenoma (APA) | 35% |
| Bilateral idiopathic hyperplasia (IHA) | 60% |
| Unilateral (primary) adrenal hyperplasia | 2% |
| Pure aldosterone-producing adrenocortical carcinoma | <1% |
| Familial hyperaldosteronism (FH) | |
| FH-I (Glucocorticoid-remediable aldosteronism) | < 1% |
| FH-II (APA or IHA) | |

1.4.1.2 The Aldosterone to Renin Ratio (ARR)

The diagnosis of PA is now largely dependent on the aldosterone to renin ratio (ARR). This index was introduced in 1981 and has since been accepted as a useful diagnostic tool for hypertensive diseases as it is relatively simple, inexpensive and can compensate for salt intake, diurnal variation and posture (Hiramatsu *et al.*, 1981; Montori and Young, 2002). A high ARR demonstrates that aldosterone production is inappropriately high for the level of renin, suggesting an error in the efficiency of corticosteroid synthesis. The test has been shown to be useful for diagnosis of PA, hypertension, hypotension, resistant hypertension (Funder *et al.*, 2008). Furthermore, the ARR is highly heritable (38.1%) thus is likely to be at least partly determined by genetic variation (Alvarez-Madrado *et al.*, 2009). To summarise, aldosterone concentration and electrolyte balance are frequently normal in PA patients, therefore the ARR can detect alterations in the corticosteroid pathway that signify an underlying pathology.

1.4.1.3 Disorders of Cortisol Production

Cortisol deficiency, for example in Addison's Disease leads to hypotension, hypoglycaemia and weight loss (Ten *et al.*, 2001). Conversely, in Cushing's syndrome, where cortisol levels are high, blood pressure is severely elevated. Other clinical examples of cortisol excess associated with hypertension are: unilateral benign or malignant adrenal adenomas or bilateral adrenal hyperplasia or dysplasia, overproduction of ACTH caused by anterior pituitary adenomas (Cushing's disease) or, rarely, by ectopic ACTH-producing tumours. It is also a risk for patient's dependant on long-term glucocorticoid therapy such as autoimmune disease and after heterogenic transplantation. The syndrome is characterised by central adiposity, glucose intolerance and hyperglycaemia, hyperlipidaemia, hypertension and severely increased risk of cardiovascular disease. In addition to its actions mediated through the GR pathologically high levels of cortisol may overwhelm the capacity of 11 β HSDII to protect the MR allowing cortisol to exert additional MR-like actions (Section 1.3.1).

1.4.1.4 Monogenic Causes of Hypertension

Another source of secondary hypertension is the mutations arising in genes encoding enzymes of the corticosteroid biosynthetic pathway. This type of Mendelian disease offers an interesting perspective on the contribution of genetics to the development of blood pressure pathology. However, these are rare, only accounting for less than 1% of hypertensive cases. To date, mutations in 8 human genes have been identified as causing hypertension and 9 as causing hypotension (Lifton *et al.*, 2001). All of these mutations result in changes in renal salt reabsorption which cause early onset, severe changes in blood pressure.

Congenital Adrenal Hyperplasia (CAH)

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive loss-of-function mutations in genes of the corticosteroid biosynthetic pathway. Defects reduce the production of cortisol therefore, stimulating massive continued ACTH production which causes hyperplasia of the cortex as well as abnormal increases in precursor levels (Section 1.2.5). Clinical symptoms result from two abnormalities, increased levels of DOC and altered levels of adrenal androgens.

The symptoms present in CAH patients depends on which enzyme the causal mutation lies. The most common mutations are in the *CYP21A2* gene coding for 21-hydroxylase which accounts for approximately 90-95% of cases of CAH; approximately 75% of patients cannot synthesise appropriate levels of aldosterone and have severe salt-wasting and hypotension (White and Speiser, 2000). As can be inferred from Figure 1-5, 21 α -hydroxylase deficiency will result in insufficient cortisol synthesis, but the resulting increase in ACTH drive will result in high levels of 21-deoxy-precursors (17-OH-pregnenolone and 17-OH-progesterone) and raised androgen synthesis, creating masculinisation in women (Krone *et al.*, 2007).

17 α -Hydroxylase deficiency comprises approximately 5-8% of CAH cases and was identified in the 1960s (Biglieri *et al.*, 1966). Inactivating mutations can be located at many positions in the *CYP17A1* gene and lead to inefficient conversion of 17-OH-pregnenolone to dehydroepiandrosterone (DHEA) (Figure 1-5). This deficiency also affects sex steroid production in the gonads and leads to

feminisation of males and failure of normal development of secondary sex characteristics in females. Massively raised DOC levels cause mineralocorticoid hypertension; high corticosterone level as a substitute glucocorticoid for cortisol.

Mutations in the *CYP11B1* gene (which codes for 11 β -hydroxylase) lead to deficient 11 β -hydroxylation, creating inefficient conversion of DOC to corticosterone and of 11-deoxycortisol to cortisol (Figure 1-5). The ratio of 11-deoxycortisol to cortisol concentration (S:F ratio) is used as an index of 11 β -hydroxylase deficiency. This form of CAH presents with mineralocorticoid hypertension and glucocorticoid (cortisol) deficiency. High androgen production leads to virilisation in girls and precocious puberty in boys.

Both 17 α -hydroxylase and 11 β -hydroxylase deficiencies cause symptoms that are associated with hypermineralocorticoidism, for example hypertension, but in this instance the causative steroid is DOC and not aldosterone. Decreased circulating cortisol concentrations associated with both deficiencies lead to an increased ACTH drive, which further increase the rate of DOC synthesis. Diagnosis is confirmed by genetic screening of the genes involved and analysing androgen concentrations. Treatment is by glucocorticoid replacement therapy which normalises ACTH drive, correcting overproduction of corticosteroid precursors and in turn, correcting the hypertension.

Glucocorticoid Remediable Aldosteronism (GRA)

GRA (familial hyperaldosteronism type I, FH-I) is an autosomal dominant disease which is characterised by high aldosterone, low renin and early onset hypertension. GRA is caused by a chimeric gene generated by unequal crossing over during meiosis at the promoter regions of the *CYP11B1* and *CYP11B2* genes. The hybrid gene contains the promoter sequence of the *CYP11B1* gene and distal regions of the *CYP11B2* gene. Thus, the novel gene is expressed in the ZF and allows ectopic aldosterone production abnormally under the control of ACTH, producing massive excess (Lifton *et al.*, 1992). Aldosterone production does not therefore respond to normal signals such as high sodium levels or expanded extracellular volume (Section 1.2.5). The unique feature of GRA is that, upon treatment with glucocorticoids, the phenotype is completely suppressed, by removing ACTH drive. A second class of familial hyperaldosteronism also exists

(FH-II) and is thought to be more prevalent than type I. Patients do not have a chimeric gene and do not respond to glucocorticoids. The molecular basis of this disease is not known but has been link to genetic variation, identified on human chromosome 7, investigations continue to identify the gene(s) involved (Jeska *et al.*, 2008).

Apparent Mineralocorticoid Excess (AME)

The syndrome of apparent mineralocorticoid excess (AME) is a rare, autosomal recessive hypertensive disorder (Ulick *et al.*, 1979). It is characterised by hypokalemia and metabolic acidosis resulting from low renin and aldosterone levels and is caused by loss of function mutations in the *11BHS2* gene, which prevents the 11 β -dehydrogenase 2 enzyme (11BHS2) from oxidising cortisol to cortisone in the kidney (Section 1.3.1). This allows cortisol to saturate the renal MR and induce a severe mineralocorticoid form of hypertension (Mune *et al.*, 1995; Stewart *et al.*, 1996). Treatment includes the use of MR antagonists, such as spironolactone. The importance of 11BHS2 is illustrated by knockout mice which exhibit severe hypertension (Kotelevtsev *et al.*, 1999).

Aldosterone Synthase Deficiency

Rare loss of function mutations in the *CYP11B2* genes impairs the final three steps of aldosterone synthesis. This rare condition causes salt-wasting and hyperkalemia, increased plasma renin activity and hypotension, with normal cortisol and sex steroids levels (White, 2004). Two forms of aldosterone synthase deficiency have been described; corticosteroid methyloxidase (CMO) type I and type II (Ulick, 1976).

In summary, PA and monogenic mutations of corticosteroid enzymes are rare in the general population and result in extreme phenotypes, but they provide irrefutable evidence that altered corticosteroid synthesis resulting from a variety of genetic changes can affect blood pressure. Recent evidence suggests that, in essential hypertension (a much more common disease) subtle genetic changes in the genes coding for corticosteroid enzymes may significantly contribute to the heritable component. This is discussed below.

1.4.2 Corticosteroids and Essential Hypertension

The following section will first outline the characteristics of the *CYP11B1* and *CYP11B2* genes and then discuss the role of these genes in essential hypertension, including the association of several polymorphisms present within these genes with raised blood pressure.

1.4.2.1 The *CYP11B1* and *CYP11B2* Genes

To recap, the *CYP11B1* and *CYP11B2* genes encode 11 β -hydroxylase and aldosterone synthase, respectively. As previously described, these gene products are crucial to the synthesis of corticosteroids and the maintenance of blood pressure. To date, only one mRNA transcript has been identified for each of the human genes; each has 9 exons and 8 introns (Figure 1-11). The genes are located in tandem on human chromosome 8 and lie only 40 kilobases (kb) apart. Both enzymes are comprised of 503 amino acids and share 95% sequence identity in coding regions and 90% in intronic regions. The 5'- and 3'- regulatory regions are the area of most heterogeneity (Mornet *et al.*, 1989). At present the 3-dimensional structure has not been solved for either protein.

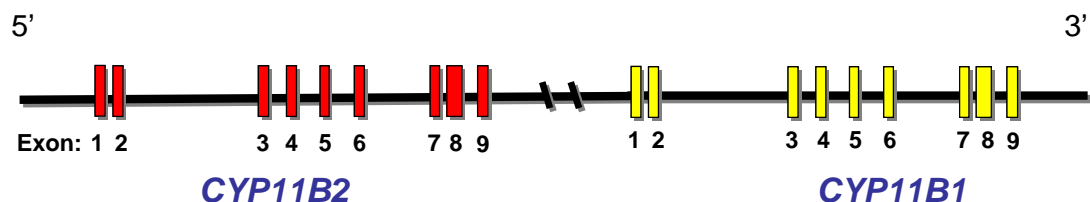


Figure 1-11. The Exon and Intron Structure of the *CYP11B1* and *CYP11B2* Genes.

Located on Human Chromosome 8. Diagram not to scale.

1.4.2.2 Genetic Variation in the *CYP11B* Locus

Several variants have been identified across the *CYP11B* locus and include SNPs, conversions and insertion/deletions. These variants are in relatively high linkage disequilibrium (LD) (a marker of the likelihood of alleles being inherited together), meaning there are few common haplotypes. One of the most commonly studied SNPs is in the *CYP11B2* promoter located at position -344 (rs1799998). This SNP lies in a binding site for the SF-1 transcription factor and can either be a C (cytosine) or T (thymine) nucleotide, in approximately equal

frequencies (Figure 1-12). A second common mutation is a gene conversion, where part of intron 2 of the *CYP11B1* gene is duplicated in the corresponding intron 2 region of *CYP11B2* (Figure 1-12) (White and Slutsker, 1995). Together, these variants are in tight LD; the frequencies of their haplotypes are : T/Conc (38%), C/Wt (45%) and T/Wt (16%) (Davies *et al.*, 1999).

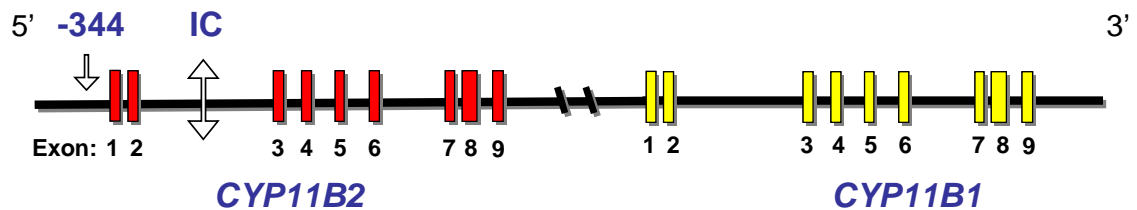


Figure 1-12. The Location of the -344 SNP and the Intronic Conversion Polymorphism.
Diagram not to scale.

Several studies in the late 1990s associated the T allele at -344 (-344T) and the presence of the intron conversion (IC) with raised blood pressure. A case-control study found that the -344T allele was more frequently identified in hypertensive patients than in normotensive controls (Brand *et al.*, 1998). However, this study failed to detect any association of the IC with hypertension possibly due to an equal frequency of this allele in both the cases and control populations (Brand *et al.*, 1998). A small study found that male subjects with the -344T allele had higher urinary aldosterone levels (Hautanen *et al.*, 1998). Moreover, Davies *et al.*, (1999) found that both the -344T allele and the IC were more frequently observed in hypertensive patients than age- and sex- matched normotensive controls. They also showed in the North Glasgow Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) study, which contained 486 individuals, there was a significant increase in urinary excretion of tetrahydroaldosterone (the main aldosterone metabolite) associated with the -344T allele of heterozygotic subjects (-344 C/T) when compared to homozygous -344C subjects (Davies *et al.*, 1999). This finding was corroborated in a separate study carried out in normotensive subjects by Paillard *et al.*, (1999) who reported significantly higher levels of plasma aldosterone concentration in -344T subjects compared to -344C subjects. A study of a small Japanese cohort identified an association between the -344T allele and low-renin hypertension, but failed to identify any difference plasma in activity or plasma aldosterone concentration (Komiya *et al.*, 2000).

However, not all reports of this type have been consistent; in one study, the -344C allele was associated with increased plasma aldosterone concentration in hypertensive patients (Pojoga *et al.*, 1998) and a large case-control study in Japanese patients failed to detect any association of the -344 SNP and the IC with hypertension (Tsujita *et al.*, 2001). A recent meta-analysis set out to address this disagreement between the studies; it confirmed the association between the -344T allele and increased risk of hypertension (Sookoian *et al.*, 2007). However, the findings were limited. It could not positively associate the -344 SNP with systolic or diastolic blood pressure or define a link between the polymorphisms and aldosterone excretion or plasma concentration (Sookoian *et al.*, 2007). These polymorphisms may have a role in PA; in 27 well-characterised Conn's patients there was a higher frequency of the -344T and IC allele (Inglis *et al.*, 2001) and the -344T allele was associated with a higher ARR in non-selected hypertensive subjects (Lim *et al.*, 2002). Despite the abundance of studies which suggest a link between the *CYP11B2* polymorphism and essential hypertension, the causative mechanism has not yet been identified. It was suggested that the -344 SNP altered the binding of SF-1 TF to the promoter to affect transcription but *in vitro*, the -344T allele has 4-fold less affinity of SF-1 and yet has no effect on gene transcription (Bassett *et al.*, 2002). There are several possible explanations; it may be the -344 and IC polymorphisms are merely markers of a causal variant in near-by genes in this region of high LD. Alternatively, the functional mutation may lie in the relatively under-studied IC region, or that other polymorphisms located across the locus may have yet undiscovered functions.

The first of these proposals that -344T/C is a marker for linked polymorphisms elsewhere has been investigated in the *CYP11B1* gene. Similar to the *CYP11B2* gene, *CYP11B1* is highly polymorphic and these polymorphisms are in tight LD with those in *CYP11B2*. It has been proposed that polymorphisms in *CYP11B1* result in inefficient 11 β -hydroxylation, identifiable by altered plasma S:F and DOC:B ratios or of urinary metabolites, tetrahydrodeoxycortisol (THS) to tetrahydrodeoxycorticosterone (THDOC) (Section 1.4.1.4). A mild 11 β -hydroxylase inefficiency, while it would not affect cortisol levels, would result in increased ACTH drive over a long time, contributing to the development of essential hypertension and mildly raised aldosterone secretion (Davies *et al.*,

2009). An argument supported by the associated increase in ARR (Lim *et al.*, 2002).

In the presence of impaired efficiency, exogenous ACTH should accentuate abnormal S:F ratio. Essential hypertensive patients given an ACTH infusion had increased S:F ratios compared to controls, supporting reduced 11 β -hydroxylation efficiency as a disease marker (Honda *et al.*, 1977; de Simone *et al.*, 1985; Connell *et al.*, 1996). This observation was then linked to polymorphisms in the *CYP11B2* gene in a study undertaken in 92 male subjects; those with the -344T/ intron 2 conversion had higher urinary aldosterone excretion rates than -344C/intron 2 wild-type subjects. Further, they had higher 11-deoxycortisol levels in plasma when stimulated with ACTH (Hautanen *et al.*, 1998). Davies *et al.*, (2001) observed a similar relationship with the polymorphisms and plasma DOC concentration in normotensive subjects and in another normotensive cohort study the excretion rate of the major 11-deoxycortisol metabolite (tetrahydro-11-deoxycortisol) being higher in urine from -344T subjects; supporting the hypothesis of inefficient 11 β -hydroxylation (Kennon *et al.*, 2004).

Other genetic variants within *CYP11B2* and *CYP11B1* genes have also been implicated in altered 11 β -hydroxylation efficiency. A large study evaluated the roles of 6 *CYP11B2* SNPs and 3 *CYP11B1* SNPs, finding a significant association between the presence of a SNP in exon 7 (T4986C) of *CYP11B2*, plasma cortisol concentrations and the S:F ratio. Additionally, a family-based study found a significant association between THaldo excretion rate, and a SNP in intron 3 (rs6387) of the *CYP11B1* gene (Imrie *et al.*, 2006). However, a complete genetic screen of the *CYP11B1* gene in normo- and hyper-tensive subjects could not relate variation in the coding region of this gene to hypertension or to an altered ARR (Barr *et al.*, 2006). Interestingly, a screen of the non-coding regions identified two novel polymorphisms in the promoter region of *CYP11B1*, at -1889 (G/T) and -1859 (A/G) (Figure 1-13), which associated with the ratio of urinary THS:total cortisol excretion. Moreover, these polymorphisms were found to be transcriptionally functional, responding differently to trophins *in vitro*. Crucially, these polymorphisms are in strong LD with the -344T/C, IC *CYP11B2* polymorphisms (Barr *et al.*, 2007).

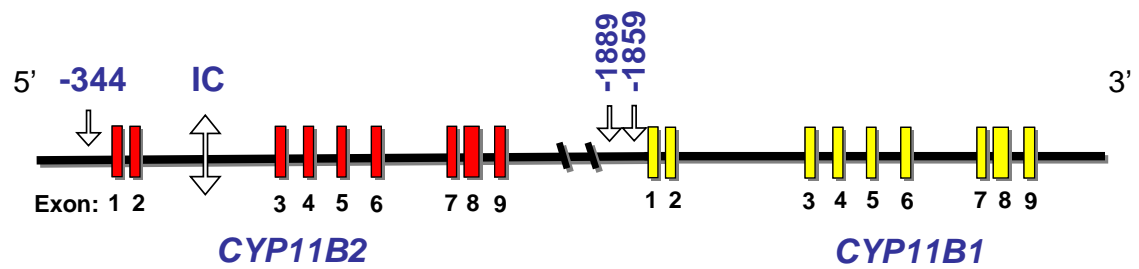


Figure 1-13. The Location of the *CYP11B1* Promoter SNPs: -1859 and -1889.
Diagram not to scale.

To summarise, genetic variation in the *CYP11B1* and *CYP11B2* genes is associated altered corticosteroid metabolism, the ARR and hypertension. The precise causal molecular modifications are uncertain but it appears likely that variation in one *CYP11B* gene can affect the other and this is supported by the strong LD identified across the locus. The foregoing discussion strongly supports the conclusion that small changes: the efficiency of corticosteroid-related enzymes, genetically-based and expressed over a lifetime, can result in clinically important changes. It seems reasonable to suggest that any other mechanism which influences the expression of the *CYP11B* genes may have a similar effect. microRNAs may be just such a mechanism, and are discussed in the following sections.

1.5 RNA Interference (RNAi)

RNA Interference (RNAi) is an evolutionarily conserved, naturally-occurring regulation mechanism in eukaryotes. Utilising small, non-coding, double-stranded (ds) RNA that is complementary to, and binds mRNA, RNAi results in post-transcriptional gene silencing. The mechanism of RNAi was first observed in plants (Napoli *et al.*, 1990) but the molecular mechanism was only fully described several years later in the nematode *Caenorhabditis elegans* (*C. elegans*) by the Melo Group (Fire *et al.*, 1998). RNAi has since been identified in many organisms. High-throughput sequencing has identified several classes of small RNA that facilitate RNAi: short interfering RNA (siRNA), micro RNA (miRNA) and p-element-induced wimpy testis RNA (piRNA or piwiRNA) (Farazi *et al.*, 2008; Kim *et al.*, 2009).

Since their discovery small RNAs have become an effective scientific tool for investigating control of gene expression which has shown them to be important regulators of cellular function with great relevance to understanding disease pathologies.

1.5.1 Short Interfering RNA

siRNAs are small double-stranded RNA molecules that bind to and silence specific mRNA targets. siRNAs can be used experimentally and introduced into cells exogenously but also can be derived endogenously by viral infection or gene transcription. siRNAs have perfect base-pairing complementarity to their target genes and result in mRNA degradation by cleavage thus, siRNAs are important regulators of various cellular functions and have become a useful research tool and offering the possibility of novel therapies (Ghildiyal and Zamore, 2009).

1.5.2 microRNAs

miRNAs are a tightly defined class of endogenous, non-coding RNA molecules that are highly conserved across evolution. The regulatory power of these molecules is a relatively new discovery and was awarded the title of scientific breakthrough of the year by 'Science' in 2002 (Couzin, 2002). miRNAs have been

identified in a wide range of eukaryotes but for the purpose of this thesis, the main focus will be on human miRNAs, unless otherwise stated.

1.5.2.1 Nomenclature

The identities and sequences of experimentally-validated miRNAs are deposited in the miRNA repository, miRBase (Griffiths-Jones, 2004;Griffiths-Jones *et al.*, 2006;Griffiths-Jones *et al.*, 2008). The database maintains accurate information on each miRNA identified thus preventing duplication. Leading scientists in the field have devised a strict naming convention for miRNAs (Ambros *et al.*, 2003). Each miRNA is preceded with a species identifier, for example, hsa (human) or mmu (mouse). miRNAs are numbered chronologically from the time of submission to miRBase and, where applicable, miRNAs with identical sequences in different species are given matching numbers. The miRNA number may be followed by a lowercase letter that identifies miRNAs with similar sequences (e.g. hsa-miR-125a and hsa-miR-125b); miRNAs with similar sequences belong to a 'miRNA family'. Two miRNAs may possibly be generated from each hair-pin pre-miRNA (Section 1.6.2.3); these are given the same number but are distinguished by -3p (3') or -5p (5') according to the arm of the hair pin from which the miRNA originated (e.g. hsa-miR-125a-3p and hsa-miR-125a-5p). Alternatively, the strands can be distinguished by an asterisk (*), representing the non-functional miRNA strand (e.g. hsa-miR-149 and hsa-miR-149*). A single miRNA type may arise from two different genetic loci and in this instance, miRNAs are given a numerical suffix to identify their origin (e.g. hsa-miR-24-1, hsa-24-2) (Ambros *et al.*, 2003).

1.5.2.2 Discovery

miRNAs were discovered in 1993, the inaugural miRNA, lin-4, was isolated from *C. elegans* (Lee *et al.*, 1993). By cloning the *lin-4* gene, Lee *et al.* (1993) established that the product was not translated into a protein but instead generated a non-coding RNA. At the same time, Ruvkun and colleagues demonstrated that lin-4 could bind to the 3'UTR (untranslated region) of the *lin-14* gene and reduce protein production (Wightman *et al.*, 1993). Further experiments showed that repression of *lin-14* by lin-4 was an important

mechanism in controlling developmental timing in *C. elegans* (Olsen and Ambros, 1999).

At the time of publication, the full significance of miRNA post-transcriptional regulation was not appreciated. However, with the discovery of the second miRNA in 2000, the importance of miRNA regulation started to become clearer. The candidate miRNA was let-7c; it was found to be highly conserved across species (Reinhart *et al.*, 2000) and to be a member of the large let-7 family, which has several subtypes. Let-7c is capable of regulating a number of developmental genes by binding to their 3'UTR and proved to be important in temporal changes in the late development of *C. elegans*. It is not expressed during early development then increases 10-fold at early pupal stage that is sustained to adulthood (Pasquinelli *et al.*, 2000).

Over the last 10 years, interest in miRNA-mediated gene regulation has accelerated and the latest update of miRBase (v. 16, September 2010) includes sequences of 1048 human miRNAs (Griffiths-Jones, 2004;Griffiths-Jones *et al.*, 2006;Griffiths-Jones *et al.*, 2008). Moreover, novel regulatory pathways have been unveiled since the discovery of miRNA (Section 1.7).

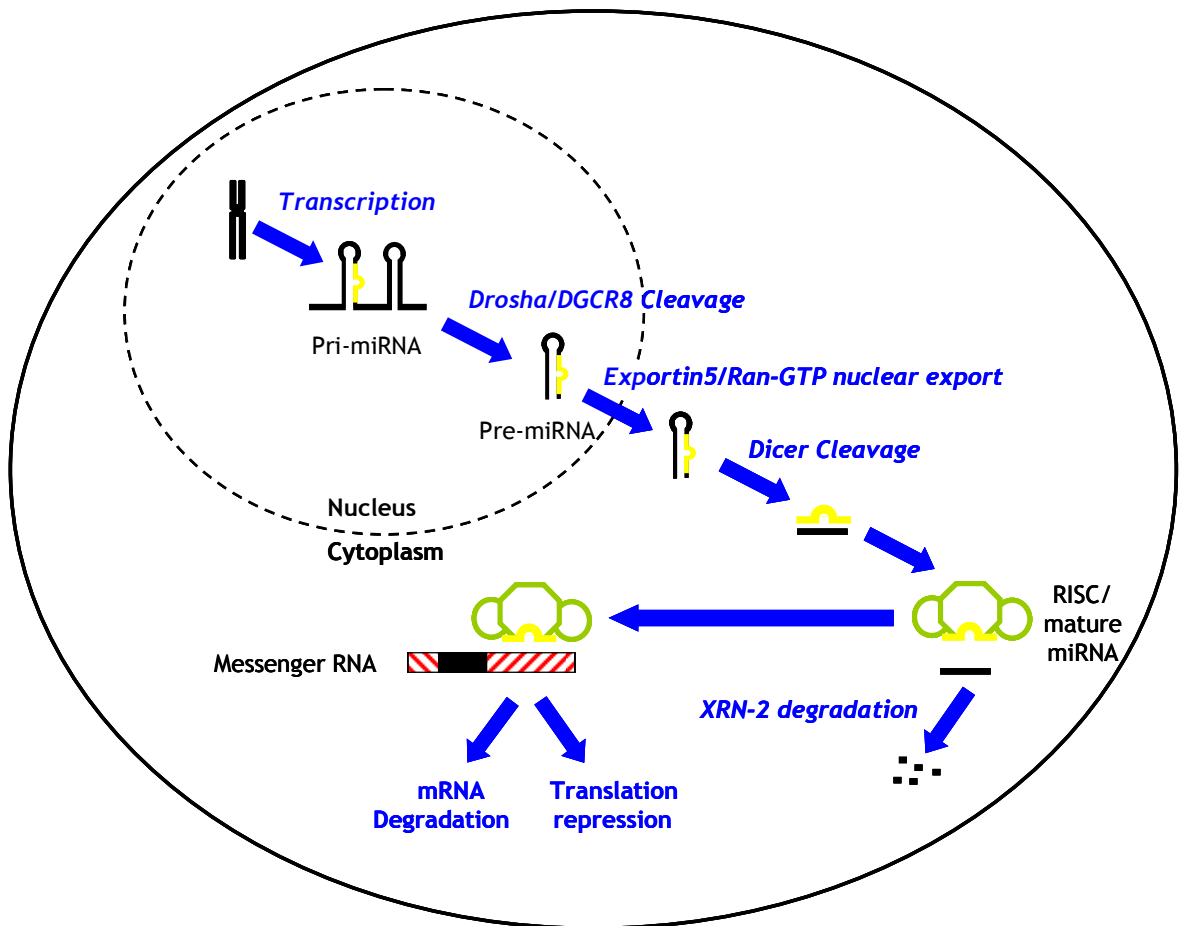


Figure 1-14. Overview of miRNA Maturation and Degradation.

Primary miRNA (Pri-miR) transcripts are transcribed in the nucleus from miRNA genes. A hair-pin structure is cleaved from the pri-miR by Drosha endonuclease. The Pre-miR is exported to the cytoplasm via Exportin-5, further cleaved by Dicer, and then unwound to form a mature miRNA (yellow). This then associates with several proteins known as the miRNA-induced silencing complex (miRISC). This then targets mRNA by binding to the 3'UTR. The passenger strand (black) is degraded by XRN-2.

1.5.2.3 Synthesis

miRNA transcription and maturation is a multistep process that starts with the nuclear transcription of a stem-loop structure from genomic DNA (Figure 1-14).

Nuclear Processing

Primary miRNA transcripts (pri-miR) are transcribed from miRNA-genes, located in intergenic chromosomal regions, by RNA polymerase II or RNA polymerase III (Lee *et al.*, 2004a; Borchert *et al.*, 2006) (Figure 1-15). Pri-miRNAs are several kilobases in length, are polyadenylated and have 5' 7-methyl-guanylate caps. Several different miRNA molecules can be excised from a single polycistronic pri-miR. miRNAs generated from the same genetic location (*i.e.* from one pri-miR) are termed a miRNA cluster. This is important as their production is subject to the same regulation (Mourelatos *et al.*, 2002) and can be important in pathophysiology for example, in lung cancer samples the expression of all six members of the miR-17-92 cluster is higher than in controls (Hayashita *et al.*, 2005).

Pri-miR transcription may be regulated by a mechanism involving ADARs (adenosine deaminase acting on RNA). ADARs catalyse the substitution of adenosine nucleotides for inosine in a process known as A-to-I editing. This was first described for both human and mouse pri-miR-22 by (Luciano *et al.*, 2004) but has been subsequently identified for other pri-miRs (Blow *et al.*, 2006; Yang *et al.*, 2006). By altering the secondary structure of a pri-miR molecule, A-to-I editing can affect miRNA processing (Section 1.5.2.3 and Figure 1-14) both enhancing synthesis (e.g. pri-miR-142; (Kawahara *et al.*, 2008) or preventing further maturation (Scadden, 2005). Thus, pri-miRs are subject to post-transcriptional regulation prior to the cleavage steps described below.

Hair-pin structures are then cropped from the pri-miR transcript. This is catalysed by a nuclear Microprocessor Complex, of which the primary components are Drosha and Di George critical region 8 (DGCR8, also known as Pasha) proteins (Figure 1-14). Drosha, an RNase III enzyme, is highly conserved between species and contains two RNase III domains and a double stranded RNA binding domain (dsRBD). DGCR8 contains two dsRBDs and is responsible for binding to the pri-miRNA and determining the site of cleavage that creates the

pre-miRNA. Other proteins involved in the microprocessor are DEAD box RNA helicases, p68 (DDX5) and p72, heterozygous nuclear ribonucleoprotein and SMAD proteins (Gregory *et al.*, 2004; Davis *et al.*, 2008). These co-factors can affect miRNA synthesis; p68 or p72 knockout mice have reduced levels of a specific subset of miRNAs (Fukuda *et al.*, 2007).

The endonucleolytic action of Drosha cleaves the pri-miRNA to produce a hair-pin structure(s), approximately 70 nucleotides in length, called pre-miRNA (pre-miR) (Figure 1-15). The pre-miR is a double-stranded hair-pin RNA molecule with regions of imperfect base-pairing along the hair-pin (Figure 1-16). Other characteristics of a pre-miRNA include a 5' phosphate group, 3' hydroxyl group and a 2-nucleotide overhang at the 3' end. The overhang serves as a recognition motif for further cleavage steps (Lund *et al.*, 2004; Zeng and Cullen, 2004). Single nucleotide polymorphisms in pri-miRNAs can prevent correct processing, for example a SNP in the mature sequence of miR-125a prevents Drosha cleavage leading to an accumulation of pri-miR-125a (Duan *et al.*, 2007). This illustrates the high degree of specificity in miRNA processing.

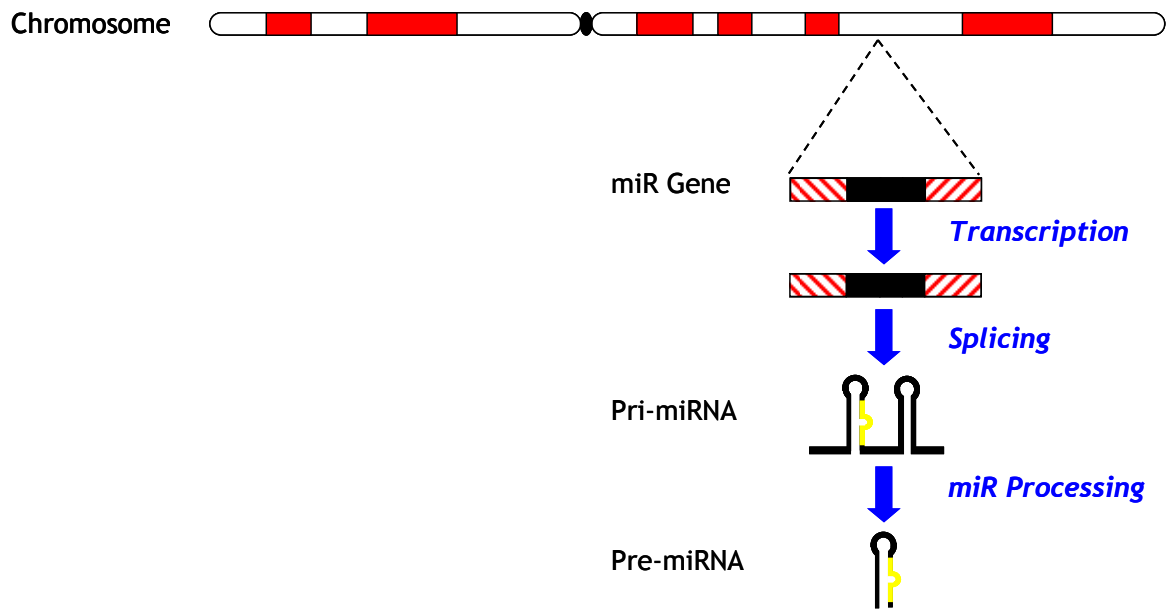


Figure 1-15. Transcription of microRNA from an Intergenic Region.

miRNA genes are located in the intergenic region (white) of chromosomes and are transcribed by RNA polymerase II to produce a primary-miRNA (pri-miRNA). Splicing produces a pri-miR. This then undergoes maturation to produce a mature miRNA.

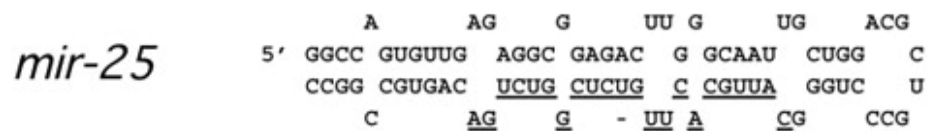


Figure 1-16. Nucleotide Sequence of a pre-miR.

An example of the nucleotide sequence for the pre-miR hair-pin of hsa-miR-25. Underlined based show the sequences of the mature miRNA. Taken from Lagos-Quintana *et al.*, (2001).

miRTrons

A Drosha-independent pathway for pre-miR production also exists in mammals, *C. elegans* and plants (Lund *et al.*, 2004; Ruby *et al.*, 2007; Okamura *et al.*, 2007; Berezikov *et al.*, 2007). miRNAs produced in this pathway are generated from miRtrons, where the hair-pin pre-miR structure is located in an intronic sequence of a protein coding gene. They are spliced out during transcription and then processed identically to those produced from the canonical pathway (Figure 1-17).

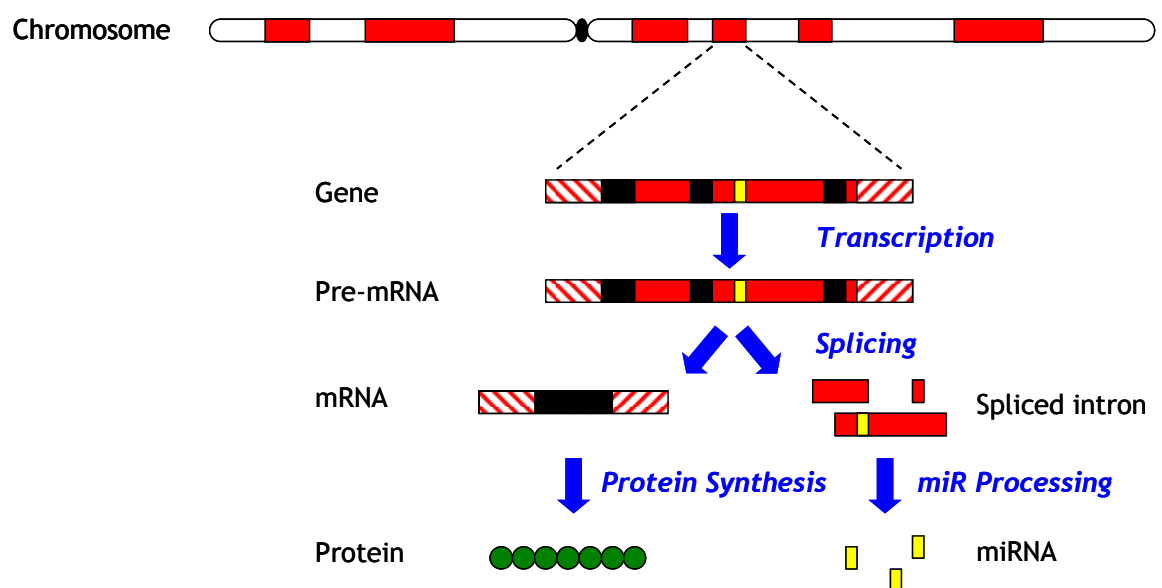


Figure 1-17. miRNAs Generated from miRtrons.

Gene transcription produces a pre-messenger RNA (pre-mRNA) strand with exons (solid red), introns (solid black) and regulatory regions (red and white stripes). Splicing removes the intron and allows mature mRNA to continue to be translated to produce protein (green). Introns are degraded but may contain a pre-miRNA (yellow), which is spliced out and can produce mature miRNAs.

Nuclear-Cytoplasm Transport

The second stage of miRNA maturation occurs in the cytoplasm (Figure 1-14). The pre-miRNA molecule is transported from the nucleus by Exportin-5 which requires Ran-GTP as a co-factor (Yi *et al.*, 2003). Exportin-5 is postulated to have quality control properties; it will only transport double stranded pre-miRs of a defined length, that have a 3' 2-nucleotide overhang (Lund *et al.*, 2004; Zeng and Cullen, 2004).

Cytoplasmic Processing

Once localised in the cytoplasm, the pre-miRNA is incorporated into the RISC (RNA Induced Silencing Complex) Loading Complex (RLC). The key components of the RLC are Dicer, an RNase III enzyme, Tar RNA binding protein (TRBP), protein activator of PKR (PACT) and Argonaut 2 (AGO2) (Gregory *et al.*, 2005; Haase *et al.*, 2005; Lee *et al.*, 2006). The pre-miR is tethered by the dsRBD of the TRBP. TRBP activates Dicer by conformational rearrangement (Ma *et al.*, 2008). Dicer cleaves the pre-miRNA at the hairpin loop of the pre-miR to produce a double-stranded miRNA, each strand of which is approximately 22 nucleotides in length. This intermediate structure contains a miRNA guide strand and a miRNA passenger strand (also known as the * strand) (Section 1.5.2.1).

The processing enzymes are essential to miRNA maturation and, as such, loss-of-function mutations in *Dicer1*, but not those in *Dicer2* prevent miRNA production (Lee *et al.*, 2004b). In contrast, depletion of PACT or TRBP only reduces the efficiency of miRNA maturation (Hutvagner *et al.*, 2001; Grishok *et al.*, 2001). Further, Dicer double knockout mice die early in development (Bernstein *et al.*, 2003) emphasising the importance of Dicer in miRNA production and of miRNA-regulation in early development. Thus, it is not surprising that Dicer levels must be closely regulated. Moreover, a product of Dicer, let-7 can repress Dicer mRNA, thus providing a negative feedback loop (Forman *et al.*, 2008). Further layers of regulation will undoubtedly be identified in coming years.

Following cleavage, Dicer and the helper-proteins dissociate from the miRNA. The final step in miRNA maturation is the unwinding of the dsRNA releasing the functional guide strand and the non-functional passenger strand. The enzyme that mediates the unwinding of miRNA has not been ascertained but there are several candidates for this roles e.g. p68 which unwinds the let-7c duplex in

mice (Salzman *et al.*, 2007); the DEAD box helicase, RCK/p54, is an alternative candidates in humans (Chu and Rana, 2006). Establishing the mechanism of miRNA unwinding and degradation (Section 1.5.2.4) will be important to understanding miRNA strand selection and expression level.

miRISC

The guide miRNA strand directs the miRNA-induced silencing complex (miRISC) to its target mRNA (Figure 1-14). The passenger strand is degraded. Whilst the full mechanism of strand selection is not known, studies with siRNA and miRNA molecules have shown that the strand with the less stable base-pairing at the 5' end of the duplex is incorporated into the (mi)RISC (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003). The passenger strand is sometimes functional and, in one example, both the guide and passenger strand of a miR species are believed to be active; co-regulation of the REST and Co-REST transcription factors, which are important in Huntington's disease, by hsa-miR-9 and hsa-miR-9*, has recently been described (Packer *et al.*, 2008).

The core component of miRISC is an Argonaut protein. Argonaut proteins are a highly-conserved, ubiquitously-expressed protein family consisting of four members in mammals: AGO1, AGO2, AGO3, AGO4. They comprise a Piwi-Argonaute-Zwille (PAZ) domain, a MID (middle) domain and a PIWI domain. Structural studies in bacteria have shown that the PAZ domain forms a pocket to associate with the 3' nucleotide overhang of the miRNA (Jinek and Doudna, 2009). The PIWI domain resembles RNase H, an endoribonuclease and therefore is believed to be important for silencing (Jinek and Doudna, 2009). Argonaut proteins also have strand selection properties but this may be more relevant to siRNA silencing (Matranga *et al.*, 2005). In mammalian systems, only AGO2 has cleavage properties and is the predominant mediator of RNAi (Liu *et al.*, 2004). *In vitro* experiments have shown that expression levels of Argonaut proteins correlate with the levels of mature miRNA, whereas their levels are independent of those of Drosha, DGCR8 and Dicer (Diederichs and Haber, 2007).

1.5.2.4 Turnover

Given the powerful regulatory role of miRNAs and their ability to modify mRNA directly, the levels of active miRNAs are likely to be closely regulated. Similar to

most cellular molecules, the abundance of miRNAs in the cytoplasm is a balance of transcription, processing and decay. Variations in miRNA levels are often associated with disease phenotypes (Section 1.7).

The regulation of miRNA degradation has a dual purpose: first to remove the passenger strand after separation of the pre-miRNA duplex and thus, preventing aberrant miRNA action, and secondly, to clear redundant guide miRNAs. Relatively little is understood about the process of miRNA degradation and the half life of miRNAs. However, recently XRN-2 exonuclease was shown to degrade single-stranded animal miRNA, both guide and passengers *in vitro* (Figure 1-14) (Chatterjee and Grosshans, 2009). Several strategies that protect against degradation have been identified, including 3' adenylation in mammals (Kato *et al.*, 2009) and 3' methylation in plants (Yu *et al.*, 2005; Li *et al.*, 2005). Moreover, miRNA abundance is correlated with the number and levels of target mRNA (Chi *et al.*, 2009) which may suggest that miRNAs incorporated into miRISC and bound to Argonaute are resistant to degradation.

In summary, miRNA synthesis, maturation and degradation are regulated, multi-step, multi-protein processes. Errors occurring during maturation, or polymorphisms in the miRNA sequence can alter the levels of miRNA and therefore have down-stream effects on target protein expression and function.

1.6 miRNA-Mediated Gene Silencing

Canonical miRNA action involves binding to the 3'UTR and consequent repression of mRNA levels, this can occur by transcriptional repression or mRNA degradation and will be discussed in section 1.6.2. Variants of this pathway have been described but it is believed that the majority of miRNAs act via this repressive mechanism.

1.6.1 miRNA Target Recognition

The sequence of miRNAs serves as a target recognition motif for guiding miRISC to target mRNA and thereby tethering the two together. miRNAs predominantly bind to the 3'UTR of mRNA, although there are examples of mammalian miRNA binding to coding regions and 5'UTR regulatory regions (Orom *et al.*, 2008; Duursma *et al.*, 2008; Lee *et al.*, 2009). Most studies, however, focus on binding to the 3'UTR and subsequent changes in target expression.

The degree of miRNA-to-mRNA base-pairing complementarity determines the fate of the targeted mRNA. Perfect base-pairing complementarity leads to mRNA endonucleolytic cleavage by AGO2, a similar mechanism to siRNA-mediated silencing. Perfect base-pairing and cleavage is the prevalent mechanism in plants but is less common in animals. In contrast, imperfect miRNA base-pairing can cause mRNA destabilisation or translational repression. The ability of miRNA to regulate, despite imperfect complementarity, is a special trait of miRNA action in animals. There are a large number of bioinformatic databases which provide researchers with *in silico* tools to predict miRNA:mRNA interaction. However, the ability of miRNAs to act even though complementarity is imperfect increases the difficulty of predicting *bone fide* targets for miRNAs accurately. This will be discussed further in Chapter 5.

1.6.2 miRNA-Mediated mRNA Repression

The original miRNA experiments, that identified *lin-14* as a target for *lin-4*, only observed repression at the protein level, changes in mRNA levels were negligible (Lee *et al.*, 1993; Reinhart *et al.*, 2000). From this, it was concluded that miRNA

regulation of mRNA expression was only apparent at the level of protein synthesis. However, repetition of these experiments (Bagga *et al.*, 2005) and subsequent studies have shown that miRNAs can reduce both mRNA and protein levels (Lim *et al.*, 2005; Guo *et al.*, 2010).

The exact mechanisms and rules governing miRNA-mediated repression are not fully understood. Current evidence suggests to two models of silencing following miRNA:mRNA target interaction: mRNA destabilisation followed by degradation or mRNA translation repression. There is strong experimental evidence in support of each model and this will be described below.

1.6.2.1 mRNA Destabilisation

miRNAs may prevent translation by destabilising mRNA which can, in turn, lead to degradation. The first step of degradation involves shortening the poly(A) tail (Wu *et al.*, 2006). Removal of the 3' poly (A) tail leaves the mRNA vulnerable to degradation and also prevents binding of PABPs (Poly (A)-binding protein) and translation initiation. mRNA degradation can then proceed in a 3'-5' manner facilitated by exosomes. Alternatively, the 5' m⁷GpppN cap is removed, typically by the decapping enzyme Dcp2, and then the mRNA is degraded in a 5'-3' direction, mediated by XRN1 (Figure 1-18) (Behm-Ansmant *et al.*, 2006).

miRNA-targeted mRNAs destined for degradation are directed to, and accumulate in, cytoplasmic foci called processing-bodies (P-bodies) (Figure 1-18)(Liu *et al.*, 2004). These were first discovered in 1997 and were implicated in miRNA silencing in 2005 (Bashkurov *et al.*, 1997; Pillai *et al.*, 2005). mRNA with shortened poly(A) tails are inducted into P-bodies (Kulkarni *et al.*, 2010). Once internalised, many degradation factors contained in P-bodies degrade the targeted mRNA. Examples of these factors were recently reviewed by Kulkarni *et al.*, (2010) and key factors are summarised in Table 1-7.

miRISC components GW182 (Protein with multiple glycine(G)-tryptophan(W) repeats and a molecular mass of 182 kD) and AGO proteins are required for mRNA de-adenylation, decapping and decay (Behm-Ansmant *et al.*, 2006). Experiments that artificially tethered GW182 to the 3'UTR of a reporter construct identified it as the key protein for decay. Thus, the current model of

miRNA-mediated decay is: miRNA targets the 3'UTR of mRNA, which is bound by the AGO of miRISC and targeted to P-bodies. AGO protein then recruits GW182, which is responsible for mRNA decay (Behm-Ansmant *et al.*, 2006; Li *et al.*, 2008).

P-bodies are motile entities that can move through the cytoplasm and also fluctuate in number and size. Decreasing XRN-1 in human cells, thus, preventing 5'-3' degradation, leads to an increase in the number and size of P-bodies (Sheth and Parker, 2003).

miRNA repression is not always irreversible. An elegant study demonstrated that the cationic amino acid transporter (CAT-1) mRNA is targeted by miR-122 (a liver specific miRNA) and directed to P-bodies (Bhattacharyya *et al.*, 2006). However, under cellular stress conditions, such as oxidative stress (induced with sodium arsenite) or endoplasmic reticulum stress (induced with thapsigargin), the mRNA can be released back in to the cytoplasm, where it can engage again in active translation. The authors demonstrated that this reversal was due to an AU-rich-element binding protein, HuR, which also bound the 3'UTR and provoked CAT-1-induced mRNA release (Bhattacharyya *et al.*, 2006). This raises the possibility that P-bodies may be temporary stores for sequestered mRNA and again emphasises the dynamic nature of miRNA 'fine-tuning' of mRNA levels.

Table 1-7. P-bodies Degradation Components and Function.

| Component | Function |
|------------|-------------------------------------|
| XNR1 | 5'-3' Exonuclease |
| GW182 | Degradation |
| Dcp2:Dcp1 | Decapping Complex |
| Rck | DEAD/H box RNA Helicase |
| Ccr4, Caf1 | 3'-5' exoribonuclease (Deadenylase) |
| AGO (1-4) | RISC Complex |

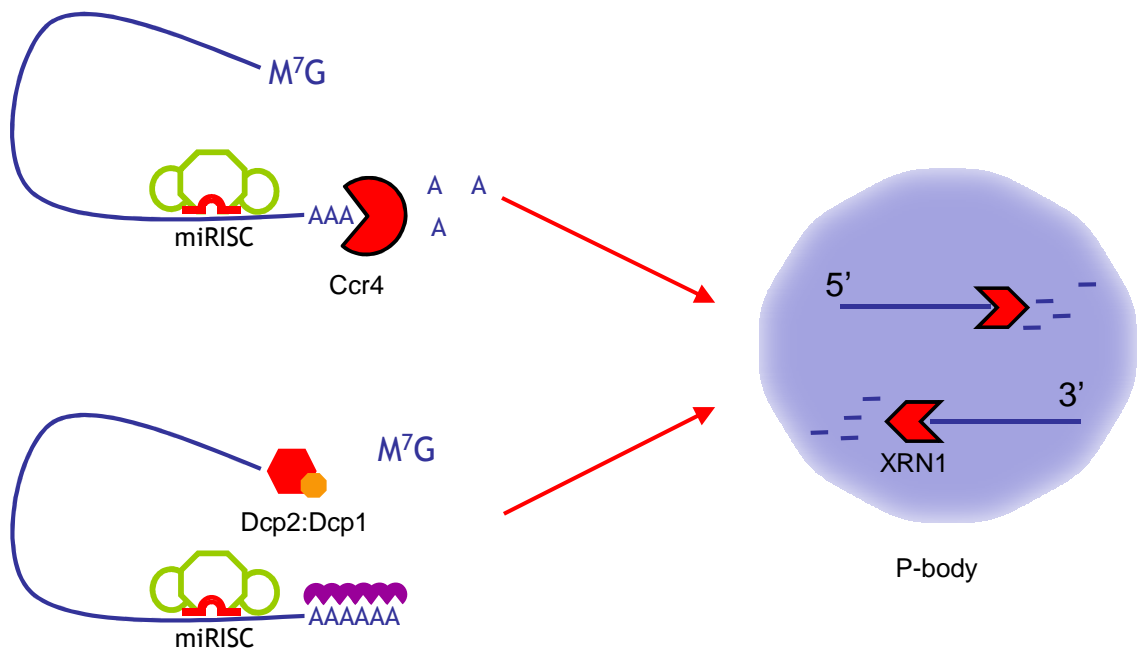


Figure 1-18. miRNA-Mediated mRNA Destabilisation.

mRNA (blue) in its pre-circularized position. miRNA (red) binds the miRNA-induced silencing complex (miRISC) (green) to the 3' untranslated region of mRNA. The top diagram shows CCR4 de-adenylating the 3' poly (A) tail. The bottom figure illustrates the Dcp2:Dcp1 complex dislodging and decapping the mRNA of its 7-methyl guanine (M7G) 5' cap. Each method leads to mRNA being targeted to cytoplasmic foci, called Processing bodies (P-bodies). Within P-bodies, mRNAs are degraded in a bi-directional action, mediated by XRN1 or other exonucleases.

1.6.2.2 Translation Repression

miRNA-mediated silencing may occur at several stages of translation. Translation is a multi-step process, transfer RNA (tRNA) is produced using the sequence of mRNA as a template. tRNA then forms the template for amino acids assembly and protein synthesis. Translation can be divided into three stages: initiation, elongation and termination. Each step must be completed for the production of a correctly aligned and folded protein. Current research seeks to establish whether miRNAs act by preventing initiation or by inhibiting other post-initiation translational steps.

The initiation step of translation begins when a ribosome recognises and binds to the 5' mRNA m⁷GpppN (where N is any nucleotide) cap. This cap is present on all transcribed eukaryotic mRNAs. Several eukaryotic initiation factors (eIFs) are required for cap recognition; there are at least 13 eIFs subtypes. They recruit the small (40S) ribosomal subunit to the 5'UTR of mRNA and interact with poly(A)-binding proteins (PABPs), present at the 3'UTR (Pelletier and Sonenberg, 1988). This interaction brings the ends of the transcript together in what is known as circularisation, which is thought to increase eIFs affinity for PABPs and increase the rate of initiation (Figure 1-19A) (Kahvejian *et al.*, 2005). The initiation process is the rate-limiting step and is under a high degree of regulatory control. In certain circumstances, mRNA translation initiation can be cap-independent. In these cases, internal ribosome entry sites (IRESs) located in the 5'UTR allow interaction with the 40S subunit (Pelletier and Sonenberg, 1988).

Initiation Inhibition

miRNA-mediated repression of translation initiation was first proposed by Humphreys *et al.*, (2005) and Pillai *et al.*, (2005) from experiments with HeLa cells transfected with reporter constructs containing known miRNA targets. They proposed that miRNAs interfere with cap recognition, therefore preventing translation initiation, and the latter study implicated cap protein, eIF4E as a target for miRNA (Figure 1-19B) (Pillai *et al.*, 2005). Several studies have demonstrated that cap-independent translation is not subjected to miRNA repression, thus modulation of cap-recognition in cap-dependent translation appears to be one method for miRNA-mediated silencing.

AGO2, a core component of the mammalian miRISC binds directly to the cap structure (Kiriakidou *et al.*, 2007). The MID region of AGO2 has a highly similar sequence to the cap-binding eIF and may competitively antagonise binding to mRNA. Further evidence of this is illustrated by mutations in the MID region which can prevent AGO2 binding (Kiriakidou *et al.*, 2007). However, the results of more recent studies disagree. One study suggested that the mutations employed by Kiriakidou *et al.*, (2007) affected interaction with GW182 proteins, thus preventing miRISC silencing (Eulalio *et al.*, 2008). Another study cast doubt on the sequence and structural similarities between the eIF and AGO2 (Kinch and Grishin, 2009).

Post-Initiation

The same group who reported the first miRNA, *lin-4* first described miRNA repression that occurs post-initiation (Olsen and Ambros, 1999). Much later, in 2006, polysomal sucrose gradients used to assess the presence of active ribosomal translation began to uncover a mechanism for repression. These studies favoured post-initiation repression that prevents successful mRNA translation during peptide elongation or at termination. However, the results of several studies seemed to indicate marginally different modes of repression. In HeLa cells, miRNA-targeted mRNA was located in active polysomes, indicating that active translation was already in progress (Maroney *et al.*, 2006). This was corroborated in experiments using the *C. elegans* *lin-41* 3'UTR targeted with let-7a in HeLa cells, and further verified using the translation inhibitor puromycin, that blocks protein synthesis. When used in this study it released let-7a-bound miRISC from polysomes therefore, confirming active translation (Nottrott *et al.*, 2006). Further, co-immunoprecipitation of an artificial N-terminal tag from miRNA-targeted mRNA in this study failed to identify nascent polypeptides, the authors concluded that miRNAs act by recruiting proteases to degrade newly synthesised peptide, termed co-translational protein degradation (Figure 1-19D) (Nottrott *et al.*, 2006).

Finally, Petersen *et al.*, (2006) demonstrated that translation already in progress could be prematurely terminated by the dissociation of the ribosome from mRNA, and that this dissociation could be caused by miRNA. Pre-mature termination of translation, termed 'ribosome drop-off', has been recently verified in a large-scale study of human and mouse cells (Figure 1-19C) (Guo *et al.*, 2010). Taken together, these studies support the concept that miRNA can repress mRNA translation post-initiation.

In summary, miRNA-mediated silencing occurs either by preventing translation or by degrading mRNA prior to translation. Whether these two methods are mutually exclusive is not yet known. Understanding how miRNAs negatively regulate mRNA is necessary for identifying miRNA targets and increases the potential for therapeutic manipulation.

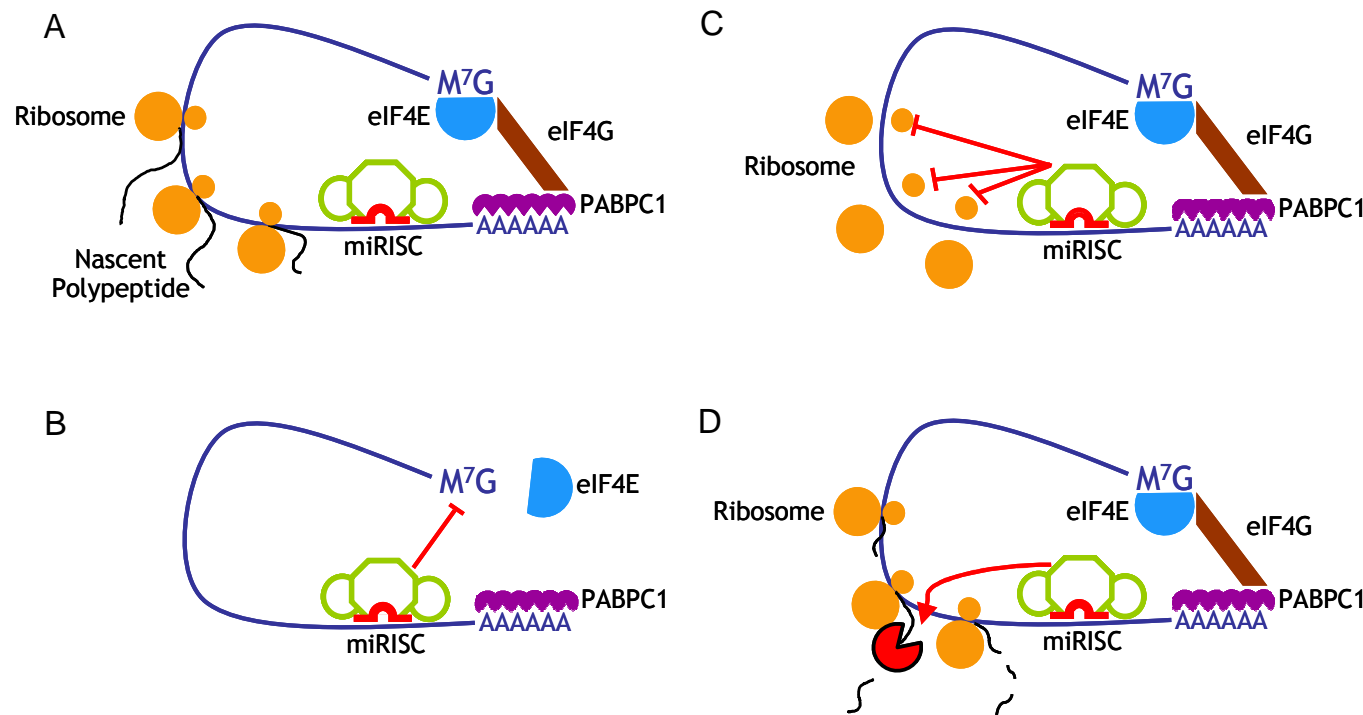


Figure 1-19. miRNA-Mediated Translation Repression: Post-initiation Mechanisms.

A – Classical transcription; mRNA (blue) in circularized position, stabilised by poly (A) binding protein C1 (PABPC1) and eukaryote initiation factors (eIF) 4e and 4G. miRNA (red) binds the miRISC (green) to the 3' UTR of the mRNA. Active ribosomes (orange) are translating mRNA to form nascent polypeptide.

B – Elongation Prevention; Indicating miRISC repression by blocking eIF4E binding to the 7-methyl guanine (M⁷G) cap, thereby preventing mRNA circularisation.

C – ‘Ribosomal Drop-off’; miRISC causing ribosome to prematurely ‘drop-off’ mRNA.

D – Co-translational protein degradation; miRISC causing nascent polypeptide to be degraded by protease (red).

1.7 Examples of Gene Regulation by miRNAs

It has been estimated that miRNAs bind and regulate approximately 30% of all genes (Lewis *et al.*, 2005) and commonly function to ‘fine-tune’ mRNA levels and protein expression rather than as ‘on-off’ switches. However, decreased expression of a miRNA or expression of *de novo* miRNAs is common in embryogenesis and development and also in several disease phenotypes. This section will focus on several examples miRNA regulating genes, pathways and diseases.

1.7.1 miRNAs and Cancer

Alterations in miRNA expression have been widely implicated in tumour development and growth. Understanding the role of miRNAs in cancer will, it is to be hoped, increase understanding of pathogenesis, allow earlier diagnosis and hopefully lead to the development of new therapies.

Altered miRNA expression as a participating factor in neoplastic disease was first observed by the Croce group investigating a chromosomal locus which is absent in chronic lymphocytic leukaemia (CLL). They showed that this region coded for two miRNAs, miR-15a and miR-16 and that the production of these was disrupted and down-regulated in 70% of CLL cases (Calin *et al.*, 2002). Sequence analysis of New Zealand Black mice, a model of CLL, identified polymorphisms in the region coding for these miRNAs thus supporting their role in the development of CLL (Calin *et al.*, 2005; Raveche *et al.*, 2007).

To date miRNAs have been implicated in many cancers including those of the lung, colon, prostate and breast; these have been reviewed by Farazi *et al.*, (2011). miRNAs implicated in cancer broadly fall into two groups based on their function: tumour suppressors or oncogenes. However, classing miRNAs in this manner has to be done carefully as they can function differently depending on the tissue or cell type in which they act. For example, miR-221 and miR-222 have contrasting functions; they can act as tumour suppressors in erythroblast cells by targeting the oncogene, KIT (Felli *et al.*, 2005), or conversely in CLL, thyroid carcinoma and hepatocellular carcinoma, miR-221 and miR-222 can

target and de-repress several tumour suppressors including, p27, p57, phosphatase and tensin homologue (PTEN) and tissue inhibitor of metalloproteinase 3 (TIMP3) (Garofalo *et al.*, 2009), thereby acting as oncogenic molecules.

Broader dysregulation of miRNAs by altered expression of biosynthetic enzymes may be important, as demonstrated by a study which measured Dicer and Drosha levels in patients with ovarian cancer (Merritt *et al.*, 2008). Low expression of these enzymes was positively correlated with disease stage, response to treatment and outcome. Given the relatively short time since the discovery of miRNAs, research has come a long way in identifying the key players in major cancers. However, much still needs to be learned and, given the heterogeneity of cancer, this may prove challenging.

1.7.2 miRNAs and Cardiovascular Disease

There is a large body of research that supports a regulatory role for miRNAs in the function of the cardiovascular system and may implicate them in cardiovascular disease. Targeted knockdown of Dicer during late development in mice leads to dilated cardiomyopathy, decreased cardiac output and heart failure; mice die prematurely at post-natal day 4 (Chen *et al.*, 2008a). Thus, miRNAs are essential for heart development and normal cardiac function.

miRNAs have also been extensively studied in a wide variety of cardiovascular disorders and have been implicated in the pathogenesis of left ventricular hypertrophy, arrhythmias and fibrosis (for a review see Small *et al.*, (2010). Several miRNAs, including miR-1 and miR-133, are cardiac-specific and appear to contribute to the regulation of a diverse range of cardiovascular functions.

Very few studies so far have investigated what role miRNAs may have in human hypertension; this is possibly due to the complex nature of hypertension. A limited number of studies have used *in vitro* methods or animal models to evaluate their role. miR-155 has emerged as a potential candidate, which may target and repress the expression of the AngII type I receptor (AT1R) (Martin *et al.*, 2006). This repression is dependent on the presence of a sequence variant A1166C (rs5186) located in the *AT1R* gene. miRNA repression of a reporter

construct was observed with the 1166A allele but not with the 1166C allele (Sethupathy, 2007). miR-155 expression is lower in aortic samples taken from spontaneously hypertensive rats (SHR) than those from control Wistar-Kyoto rats, moreover in these samples there was decreased protein levels of the AT1R (Zheng *et al.*, 2010). Repression of AT1R by miR-155 would be expected to decrease the vasoconstrictive effects of angiotensin II, thus lowering blood pressure. Hypertension has been associated with the 1166C allele in some populations but not others; if there is a link with the 1166C and hypertension, it would be expected that patients with the 1166C allele would have reduced miR-155 repression of AT1R and, therefore, greater AngII signalling. SNPs present in the genes of other RAS components may similarly modulate the effects of miRNA regulation (Elton *et al.*, 2010).

Thus, whilst miRNAs have distinct expression patterns in cardiovascular tissue and may have major roles in heart development and disease progression (including hypertension-related cardiac pathology), their role in hypertension has not yet been incontrovertibly established.

1.8 Therapeutic Potential of miRNAs

Novel therapeutic strategies are always being sought for diseases, either to improve existing treatment (e.g. less invasive, more specific and manageable strategies), or to treat conditions that do not respond to standard treatment. Unsurprisingly, much research time and money has been devoted to identifying ways of manipulating miRNAs, either by increasing or decreasing their expression as the basis of novel therapies. Problems to be solved include the methods of delivery to target tissues, synthesising stable analogues and reducing off-target effects.

Current synthetic miRNA therapies include the use of viruses to generate pre-miR molecules (Kota *et al.*, 2009), or direct administration of nucleotide analogues that act as mature miRNAs (Figure 1-20). Several companies are concentrating on making these mRNA-mimics as short as possible in order to increase specificity; RXi Pharmaceuticals (Worcester, MA) are investigating the

potential of 15-nucleotide-long dsRNA molecules, whereas Santaris Pharma (Hoersholm, DK) have tested molecules as short as 8 nucleotides.

Options for reducing the destructive effects of miRNA broadly fall into three areas that are summarised in Figure 1-20. They include direct inhibition of the miRNA using antisense molecules, sequestering miRNA with a miRNA-sponge (Ebert *et al.*, 2007), which has several repeats of 100% complementary miRNA sequences, or antagonising miRNA binding sites in the 3'UTR of mRNA (Figure 1-20).

Mechanisms for miRNA delivery include viral vectors or short synthetic oligonucleotides which include the miRNA sequence (Figure 1-20). While viral vectors can provide long term treatment, they also require accurate targeting to the cell nucleus (Section 1.5.2.3). Synthetic RNA molecules are easier to deliver to the cytoplasm but have very short half-lives. For this reason, several strategies that modify nucleic acid structure, helping to stabilise them have been devised (Figure 1-21). Modifications include addition of a methylene bridge between the 2'-O atom and 4'-C atom of the ribose ring. Termed Lock Nucleic Acid (LNA), this can increase the hybridisation affinity to ssRNA (Vester and Wengel, 2004). Another modification introduces a 2'-O-methyl group to the nucleotide which by reducing nuclease degradation, increases stability and also increases affinity for specific RNA molecules (Hutvagner *et al.*, 2004). Finally, a phosphorothioate linkage can be modified; this involves the replacement of the oxygen molecule that normally links a nucleotide to its adjacent nucleotide with a sulphur molecule. Again, this increases stability and reduces plasma clearance; however, it also reduces the affinity for the target RNA. That these approaches are promising is shown by the fact that anti-miRs with both a 2'-P-methyl group and phosphorothioate link successfully target and repress mature miR-122 in the liver for up to 23 days following injection into the tail vein of mice (Krutzfeldt *et al.*, 2005); this was the first effective use of antisense miRNA molecules *in vivo*.

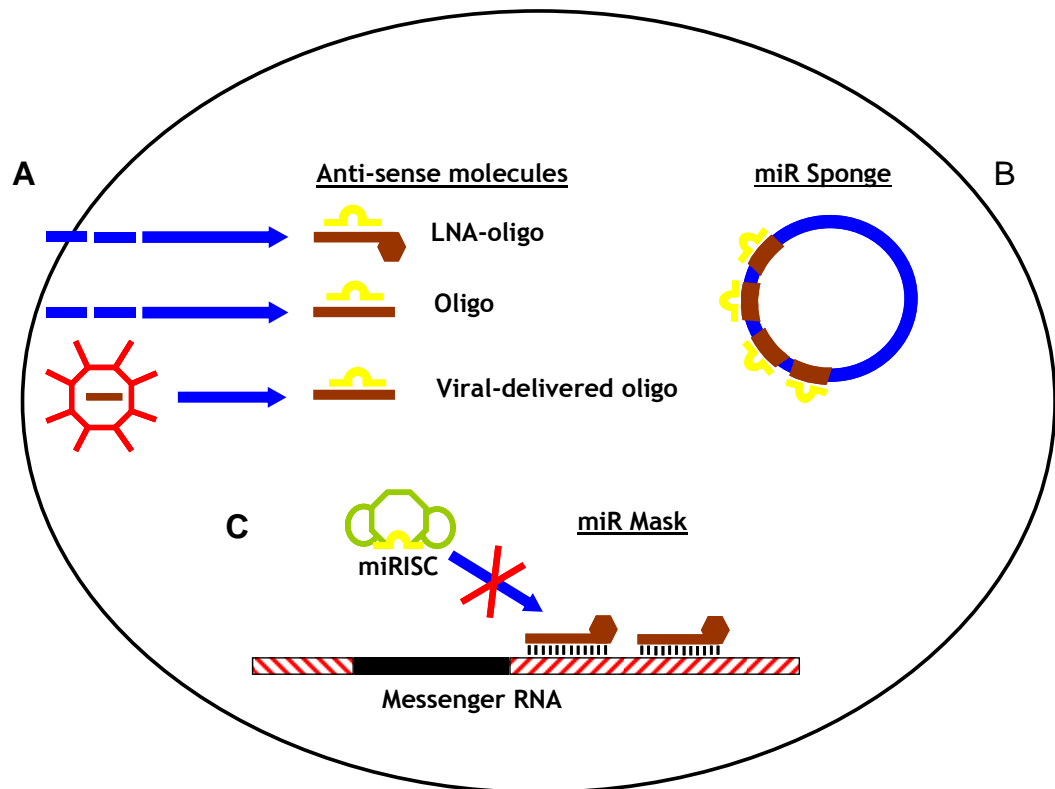


Figure 1-20. Methods of Inhibiting miRNAs.

A - miRNA inhibition by small anti-sense molecules, which bind to the miRNA molecules, thus preventing them from acting. Delivery mechanisms include small oligonucleotide, with or without modification, or by viruses. B - miR-sponge is a reporter construct with several repeats of the antisense miRNA sequence; these bind miRNAs and inhibit them. C - miR-masking inhibitors are small molecules which competitively antagonise the miRNA binding site present on the 3'UTR.

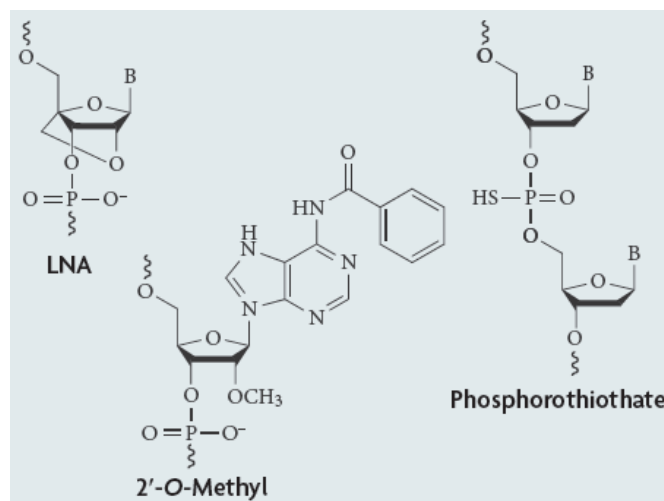


Figure 1-21. RNA Structural Modifications.

Examples of modifications to nucleotides which stabilise small RNAs. Locked Nucleic Acid (LNA) involves adding a methylene bridge between the 2'-O atom and 4'-Carbon atom of the ribose ring. 2'-O-Methyl modification introduces a 2'-O-Methyl group to the nucleotide. A phosphorothioate link replaces the oxygen molecule on the nucleotide with sulphur. Taken from Garzon *et al.*, (2010).

1.8.1 Current Clinical Trials

Despite the challenges described in the previous section, there are several pharmaceutical companies who are conducting early pre-clinical trials of miRNA-based therapy. The *in vivo* delivery in small mammals could be replicated in higher mammals was first demonstrated by Elmen *et al.*, (2008), they successfully targeted the liver of African green monkey with a LNA anti-miR-122. Subsequently, several pharmaceutical companies have lodged patents for miRNA therapies and Santaris Pharma this year announced that they are commencing phase II clinical trials using a similar anti-miR-122 (SPC3249) as a treatment for Hepatitis C Virus. Several other companies (and presumably academic institutes) are actively pursuing similar therapies and, thus, the search for new treatments based on miRNAs appears highly promising.

1.8.2 miRNAs as Biomarkers

Further clinical benefits of miRNAs lie in their potential as biomarkers, providing diagnostic signatures of disease. Biomarkers aim to be non-invasive, routine identifier of disease phenotype, stage and response to treatment. DNA is routinely recovered from plasma and, more recently, techniques to isolate RNA (both coding and non-coding) from plasma have opened the door to their use as novel biomarkers (Lawrie *et al.*, 2008). miRNAs have also been successfully isolated from urine, saliva and amniotic fluid (Chen *et al.*, 2008b). The pattern of miRNAs in biological fluids is remarkably reproducible between patients (Chen *et al.*, 2008b) and as they are relatively unharmed by enzyme degradation, freeze-thawing and pH changes, they survive isolation unchanged (Mitchell *et al.*, 2008).

Recent analysis of blood samples from patients with non-small cell lung carcinoma revealed a sub-set of 24 miRNAs whose expression levels were different from those of healthy controls. miRNA levels could be measured with greater than 95% accuracy and specificity and with greater than 90% sensitivity (Keller *et al.*, 2009). Another study examined plasma from patients with colorectal cancer and identified 5 miRNAs (out of 95 miRNAs screened) were up-regulated relative to healthy controls (Ng *et al.*, 2009). The same 5 miRNAs were

up-regulated in cancerous tissue compared to adjacent non-cancerous tissue (Ng *et al.*, 2009).

These examples indicate the great potential of miRNAs to act as a diagnostic tool in disease.

1.9 miRNAs and Endocrine Disease

The only published report of miRNAs modulating aldosterone production was published in 2007 and identified an affect of miR-21 (Romero *et al.*, 2008), which is expressed in the adrenocortical cell line, H295R, and is significantly up-regulated in response to AngII stimulation. H295R cells are of cancerous origin and miR-21 has previously been implicated in oncogenesis (for review see Krichevsky and Gabriely (2009)). Experimental over-expression of miR-21 in these cells increased aldosterone production but not cortisol (Romero *et al.*, 2008). Unfortunately, the investigators failed to assess the effect of miR-21 on *CYP11B2* mRNA or other known regulators of aldosterone biosynthesis. Although this important study suggests a role for miRNAs in corticosteroid production, further work is warranted.

As discussed in section 1.7.1 miRNAs are frequently discordantly expressed in tumours, and this applies also to endocrine tumours. Of particular relevance to this thesis are two recent profiling studies, one of which profiled normal adrenal glands, benign adenomas and adrenocortical carcinoma (ACC) (Tombol *et al.*, 2009), and another which compared macronodular adrenocortical disease samples with normal control tissue (Bimpaki *et al.*, 2010). These studies used Taqman Low Density Arrays to measure the expression of 365 miRNAs, less than a third currently of the total known human miRNA species. The expression of several miRNAs was altered in the diseased tissue compared to the control tissue but neither of these studies was designed to assess miRNA regulation of the *CYP11B1* or *CYP11B2* genes directly.

Further evidence that miRNAs may be determinants of adrenal function comes from a recent study in which SF-1 positive cells (adrenal, ovary and testis cells) were selectively targeted for Dicer knockout by Cre- mediated (Huang and Yao, 2010). While embryonic development of all SF-1 organs proceeded as normal, at

18.5 days post-coitum, adrenal cortex cells became apoptotic and, 48 hours later, the primordial adrenal gland degenerated. It is interesting to note that the testes were affected in a similar manner but the ovaries were normal for the duration of the study, until post-natal day 5 (Huang and Yao, 2010). This emphasises the absolute necessity of miRNAs in late adrenal development and organ maintenance.

The review of recent studies makes clear that miRNAs are important in adrenal development and pathology, and provide preliminary evidence for miRNA-mediated regulation of genes involved in corticosteroid biosynthesis. Moreover, several groups have reported the regulation of steroid receptors including MR (Sober *et al.*, 2010) and others by miRNAs (for review see Tessel (2010)). miRNA involvement in the regulation of adrenocortical function is becoming established, adding another facet to an already complex system of control. A further understanding of its precise contribution will require careful dissection of its involvement at the important steps of corticosteroid synthesis and function, as described in the earlier sections of this review.

1.10 Aims

The influence of the *CYP11B1* and *CYP11B2* genes in hypertension is well established. However, much still needs to be understood about the regulation of these genes and the consequential impact on aldosterone and cortisol production. Both genes are highly polymorphic (Section 1.4.2.1). The link between these polymorphisms and enzyme activities is yet to be fully elucidated. Finally, the processes underlying the development of APA are still unclear. The novel regulator miRNA may provide some explanations will be investigated in this study.

The aims of the study were:

To investigate the 3'UTR of the *CYP11B1* and *CYP11B2* genes in order to identify putative miRNA binding sites and also the existence and location of polymorphic variation in the 3'UTR.

To establish the expression profile for miRNAs in normal adrenal glands and in aldosterone-producing adenoma samples.

To develop methods to determine the identity and effect of miRNAs acting on *CYP11B1* and *CYP11B2* mRNA.

To determine using *in vitro* systems, whether miRNAs regulate the corticosteroidogenic pathway.

2 Material and Methods

2.1 Cell Culture

Culturing of cell lines was performed in a biological class II vertical laminar flow cabinet using sterile conditions. Cells were grown in a monolayer in tissue culture flasks, with normal growth medium placed in a humidified chamber at 37°C in 5% CO₂/95% air.

HeLa cells (an immortalised cell human line derived from cervical cancer) were purchased from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, U.K.). HeLa cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L- glutamine and 1 IU penicillin, 100 µg/ml streptomycin (Invitrogen, Paisley, U.K.).

The H295R (strain 2) human adrenocortical tumour cell line was a kind gift from Professor William Rainey (Medical College of Georgia, GA, U.S.A.). H295R cells were maintained in Dulbecco's modified Eagles medium with F12 supplement (Invitrogen, Paisley, U.K.) containing HEPES buffer, L-Glutamine and pyridoxine HCl medium. This was supplemented with 2.5% Ultrosor G (Pall Bioscience, France), 1% insulin-transferrin-selenium (ITS) (BD Biosciences, Paisley, U.K.), 1 IU penicillin, 100 µg/mL streptomycin (Invitrogen, Paisley, U.K.).

2.1.1 Cryopreservation

For long-term storage, cells were preserved by collection as described in section 2.1.3. After centrifugation, cells were resuspended in complete growth medium, supplemented with 10% (v/v) dimethyl sulphoxide (DMSO) to prevent ice crystals forming during the freezing process. Cells were aliquoted into cryopreservation vials, which were cooled at a constant -1°C/minute to -80°C using isopropanol and a Nalgene Cryo-Container and then placed into the vapour phase of liquid nitrogen for storage.

2.1.2 Revival of Cell Stocks from Liquid Nitrogen

To revive cells from cryopreservation, vials were quickly thawed in a 37°C water bath and the cell suspension immediately transferred to a universal container with 10 ml of complete growth medium. The suspension was centrifuged at 478 x

g for 5 minutes to remove the DMSO. The cell pellet was resuspended in 5 ml of complete growth medium, transferred to a T-25 cell culture flask and cultured, as described in section 2.1.3.

2.1.3 Sub-Culturing Cells

Cells were sub-cultured when approximately 70-80% confluent. The medium was removed and the cells were washed with 10 ml of phosphate buffered saline (PBS), which was also discarded. To detach the cells from the flask, 5 ml of 0.025% Trypsin-EDTA solution was added to the cells and the flask placed into the incubator for approximately 5 minutes. Once the cells were successfully dispersed, 5 ml of normal growth medium was added to inactivate the Trypsin-EDTA. The cell suspension was placed in a 25 ml universal container and centrifuged at 478 x g for 5 minutes. The supernatant was discarded and the cell pellet resuspended in an appropriate volume of normal growth medium for sub-culturing. Typically H295R cells were sub-cultured at a ratio of 1 in 3, and HeLa cells at 1 in 8.

2.1.4 Counting Cells

Counting of cells was carried out using a Bright Line Haemocytometer (Sigma-Aldrich, Poole, U.K.). Cells were collected using Trypsin-EDTA, as described in section 2.1.3. After centrifugation, cells were resuspended in 1 ml of complete growth medium. A coverslip was placed on the haemocytometer and 20 µl of cell suspension applied by capillary action across the chamber. Using a light microscope, the number of viable cells in each 1 mm corner square was counted and the average calculated. Each corner square on the haemocytometer, with coverslip in place, represents a total volume of 1 mm³ (equivalent to 10⁻⁴ cm³). Since 1 cm³ is equivalent to a volume of approximately 1 ml, the subsequent concentration of cells per ml can be determined using the following calculation:

$$\text{Cell number /ml} = \text{average cell count per square} \times 10^4 \times \text{original volume}$$

The calculated cell concentration (cell/ml) was then used for plating cells at the desired density.

2.1.5 Stimulation of Cells with Angiotensin II

CYP11B2 gene transcription was stimulated in H295R cells by supplementing the medium with Angiotensin II (AngII) (Sigma-Aldrich, Poole, U.K.). Normal growth medium was supplemented with AngII at a final concentration of 10 nM by adding 150 µl of AngII (10 µM stock) to 150 ml of growth medium and added to the cells for 1, 6 or 24 hours.

2.2 Total RNA Isolation

Specialised methods preserving the small RNA fraction were used to isolate total RNA from cells and tissue. The miRNeasy mini kit (QIAGEN, Crawley, U.K.) was used to isolate total RNA from cells and frozen tissue samples because it facilitates the purification of both large and small (<200 base pairs) RNA molecules. The RecoverAll™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Warrington, U.K.) was used to isolate total RNA from formalin-embedded paraffin-embedded tissue. RNase free plastics, reagents and handling techniques were implemented when working with RNA to minimise degradation of sample.

2.2.1 Total RNA Isolation from Cells and Frozen Tissue

For isolation of total RNA from cells, the direct lysis method of the miRNeasy Kit was followed. The cell medium was removed and the cells washed twice with PBS before adding 700 µl of Qiazol reagent directly to the cells in order to homogenise them. The Qiazol lysis reagent contains phenol and guanidine thiocyanate which efficiently lyse the cells and inhibit RNases. The cell culture vessels were placed in the -80°C freezer for at least 15 minutes to facilitate lysis then thawed at room temperature. The sample was then transferred to an RNase-free 1.5 mL tube and left at room temperature for 5 minutes.

For frozen adrenal glands, a 3 mm³ sample (approximately 40 mg of tissue) was placed into a FastPrep Lysing Matrix D Tube (MP Biomedicals, Illkirch, France) with 700 µl of QIAzol lysis reagent. Samples were homogenised in a bench top MagNA Lyser instrument (Roche Applied Science, Indianapolis, USA) for 30 seconds at a speed of 535 rpm. The tube was briefly centrifuged to collect the

contents, which were then transferred to an RNase-free tube and left at room temperature for 5 minutes.

Following this incubation, 140 µl of chloroform was added and the tube shaken vigorously for 15 seconds, then incubated at room temperature for 3 minutes. To separate the sample into aqueous and organic phases, the samples were centrifuged at 4°C, 12,000 x g for 15 minutes. The upper, aqueous phase, containing the RNA fraction, was transferred to a clean tube and 1.5 volumes (approximately 425 µl) of 100% molecular grade ethanol added to each sample. The sample was then loaded into an RNeasy mini spin column which was centrifuged for 30 seconds at 8,000 x g to bind RNA molecules within the sample to the silica membrane. The flow-through was discarded and then the membrane washed with 700 µl of Buffer RWT. This was followed by two further washes with 500 µl of Buffer RPE, the first was centrifuged at 8,000 x g for 30 seconds and the second for 2 minutes. The spin column was then placed in a fresh collection tube and centrifuged at 13,000 x g for 1 minute to prevent carry-over of wash buffers. The column was then placed into a 1.5 ml tube, 50 µl of RNase-free water added to the centre of the membrane, left to stand for 1 minute and centrifuged at 8,000 x g for 1 minute to elute the RNA.

RNA was quantified using the Nanodrop (section 2.2.4), DNase treated (section 2.2.3) prior to qRT-PCR measurement, and stored at -80°C.

2.2.2 Total RNA Isolation from Formalin-Fixed Paraffin-Embedded Tissue

The RecoverAll™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Warrington, U.K.) was used to isolate total RNA from adrenal gland samples that had been formalin-fixed and paraffin-embedded. Four 20 µm sections of tissue were cut using a microtome by Tim Harvey (University of Glasgow, Department of Pathology) and placed in a FastPrep Lysing Matrix D Tube (MP Biomedicals, Illkirch, France) with 1 ml of xylene. The samples were placed in a benchtop MagNA Lyser instrument (Roche Applied Science, Indianapolis, USA) for 30 seconds at a speed of 535 rpm to homogenise the sample. The matrix tube was briefly centrifuged to collect the contents, which were then transferred to an

RNase-free 1.5 mL tube and placed in a 50°C heat block for 3 minutes to melt the paraffin.

The sample was separated into a tissue pellet and a xylene upper layer by centrifugation at maximum speed for 2 minutes. The xylene layer was discarded. Two washes with 100% ethanol were then carried out to remove residual traces of xylene and to aid drying of the tissue. Ethanol was carefully removed and discarded each time. The pellet was then left at room temperature for 40 minutes to air dry. 100 µl of digestion buffer and 4 µl of protease solution (both supplied in the kit; concentration unknown)) were added to each sample tube, gently mixed and then placed in a heat block for 15 minutes at 50°C, which was increased to 80°C for a further 15 minutes.

The sample was cooled to room temperature prior to adding 120 µl of Isolation Additive and 275 µl of ethanol. The solution was mixed by pipetting, then applied to a filter cartridge, placed in a collection tube, was centrifuged at 10,000 x g for 30 seconds, the flow through was discarded. One wash step with 700 µl of Wash 1 and one with 500 µl of Wash 2/3 was performed, centrifuging at 10,000 x g for 30 seconds each time. A final spin of 30 seconds was performed to remove residual fluid from the filter.

An on-column DNase reaction was performed by combining 6 µl of DNase buffer, 4 µl of DNase solution (supplied in the kit) and 50 µl of Nuclease-free water, then adding this solution to the cartridge. The filter was incubated for 30 minutes at room temperature. The two previous wash steps were repeated, this time with a final spin for 1 minute at 10,000 x g. The filter was transferred to a new RNase-free 1.5 mL tube and 60 µl of nuclease-free water added. The sample was incubated at room temperature for 1 minute before eluting the RNA at 10,000 x g for 1 minute. The sample quality was verified by Nanodrop (section 2.2.4) and Bioanalyser (section 2.2.5) and then stored at -80°C.

2.2.3 DNase Treatment of RNA

DNase treatment was performed to remove residual genomic DNA from RNA samples, thus ensuring qPCR measured only mRNA. DNase treatment was performed using the TURBO DNA-free™ kit (Ambion, Texas, USA). For a 50 µl RNA

sample, 2 µl of TURBO DNase (2U/µl) and 5 µl of 10X TURBO DNase Buffer was added and carefully mixed. The reaction was incubated at 37°C for 30 minutes. To inhibit the reaction, 10 µl of DNase Inactivation reagent was added, mixed and left at room temperature for 2 minutes. The samples were then centrifuged at 10,000 x g for 1.5 minutes. The supernatant, containing RNA, was collected and stored in an RNase-free tube at -80°C.

2.2.4 Determination of Nucleic Acid Quantity

A Nanodrop® ND-100 Spectrophotometer with ND-1000 v3.1.0 software (Labtech International Ltd, Lewes, East Sussex, U.K.) was used to determine the quantity and quality of nucleic acids. The Nanodrop® was set to measure DNA or RNA as appropriate. Nuclease-free water was used to blank the Nanodrop prior to measuring samples. To load the pedestal, 2 µl of water or sample was applied and the arm lowered. The arm automatically moves to pull the sample into a column between it and the pedestal, which creates an optimal path length for the sample to be measured. The concentration of the sample is calculated using the Beer-Lambert Law of absorption;

$$\text{Concentration of DNA (}\mu\text{g/ml)} = (A_{260} \text{ reading} - A_{320} \text{ reading}) \times 50$$

$$\text{Concentration of RNA (}\mu\text{g/ml)} = (A_{260} \text{ reading} - A_{320} \text{ reading}) \times 40$$

The ratio of absorption at 260 nm and 280 nm was used as an indicator of purity of DNA and RNA samples, with ratios of 1.8 and 2, respectively being indicative of pure samples. Additionally, the ratio of absorption at 260 nm and 230 nm was used as an indicator of RNA purity; pure RNA has a ratio of 2.0 - 2.2, (a common contaminant is phenol, which absorbs at 230 nm and is used in Total RNA extraction).

2.2.5 Agilent Bioanalyser

Total RNA samples isolated for microarray analyses were tested for degradation using an Agilent Bioanalyser 2100 and a Eukaryote Total RNA Nano Series II chip. The analysis was run at the Molecular Biology Support Unit at the University of Glasgow. Results produced included an electropherogram, with RNA peaks at 18S and 28S, and a RNA integrity number (RIN).

2.3 miRNA Microarray

To determine the miRNA expression profile of adrenal tissue (non-tumourous 'normal' and aldosterone-producing adenoma) total RNA was analysed on a miRNA microarray. This was performed by LC Sciences (Houston, Texas, U.S.A.) using 5 µg of the total RNA previously prepared. For shipping, 0.1 volume of 3M NaOAc (pH 5.2) and 3 volumes of 100% ethanol were added, mixed and placed in an RNase-free 1.5 mL tube. The samples were packed in dry ice and ice blocks for shipping.

Samples were further quality checked prior to proceeding with the miRNA µParaflo® technology microarray, version 10.1. Briefly, the RNA was labelled with a fluorescent dye then hybridised overnight onto a microfluidic chip containing complementary probes for 723 human miRNAs and other RNA controls. The chip was imaged using a GenePix 4000B laser scanner and digitised using Array-Pro image analysis software. Raw data matrix was then subtracted from the background matrix. Data adjustment included data filtering, Log₂ transformation, gene centring and normalisation.

2.4 Reverse Transcription

First strand complementary DNA (cDNA) was produced from RNA by reverse transcription to allow quantification of miRNA and mRNA by qRT-PCR. Three different protocols were followed to achieve this depending on the nature of the experiment and each is detailed below. For each RNA sample a no template and a no reverse transcriptase (-RT) control was included to assess the specificity of the qRT-PCR reaction to detect genomic DNA.

2.4.1 *ImProm-II™ Reverse Transcription System*

The ImProm-II™ Reverse Transcription System (Promega, Madison, WI, U.S.A.) utilises random primers to reverse transcribe mRNA transcripts in the sample. Following the manufacturer's instructions, 200 ng of DNase treated RNA was combined with 1 µl of Random primers (0.5 µg/reaction) and made up to a final volume of 5 µl with RNase-free water in a 96-well plate. The samples were

heated to 70°C for 5 minutes on a 96-well Dyad Disciple™ thermal cycler (MJ Research, Waltham, M.A, U.S.A.), then placed on ice for 5 minutes. The following reagents were combined in a master mix, mixed, centrifuged and then 15 µl of the master mix added to each sample.

| Reagent | Volume (µl) | | Final Concentration |
|----------------------------------|-------------|-----|---------------------|
| | +RT | -RT | |
| 5X ImProm-II™ Reaction Buffer | 4.0 | 4.0 | 1X |
| dNTP Mix | 1.0 | 1.0 | 0.5 mM/dNTP |
| MgCl ₂ | 4.8 | 4.8 | 6 mM |
| RNasin Ribonuclease Inhibitor | 0.5 | 0.5 | |
| ImProm-II™ Reverse Transcriptase | 1.0 | 0.0 | |
| RNase-Free Water | 3.7 | 4.7 | |

The samples were subjected to the conditions below on a 96-well Dyad Disciple™ thermal cycler (MJ Research, Waltham, M.A, U.S.A.) then 80 µl of nuclease-free water added to each sample, which was then stored at -20°C until required.

| Step | | Time (minutes) | Temperature (°C) |
|------|----------------------|----------------|------------------|
| 1 | Annealing of primers | 5 | 25 |
| 2 | Extension | 60 | 42 |
| 3 | Inactivation of RT | 70 | 95 |

2.4.2 TaqMan[®] microRNA Reverse Transcription

TaqMan[®] MicroRNA Reverse Transcription Kit (Applied Biosystems, Warrington, U.K.) was used to generate cDNA of specific miRNAs or controls, to be used with Taqman[®] MicroRNA Assays. A reverse transcription master mix was prepared as follows;

| Reagent | Volume (µl) | |
|--|-------------|-------|
| | +RT | -RT |
| 10X Reverse Transcription Buffer | 0.750 | 0.750 |
| dNTP Mix (100 mM) | 0.075 | 0.075 |
| RNase Inhibitor (20 U/µl) | 0.094 | 0.094 |
| MultiScribe [™] Reverse Transcriptase (50 U/µl) | 0.500 | 0.000 |
| TaqMan [®] MicroRNA assay primer | 1.500 | 1.500 |
| RNase-Free Water | 3.581 | 4.081 |

The reverse transcription master mix was added to 2 µl of DNase treated Total RNA (5 ng/µl). The plate was placed on a 96-well Dyad Disciple[™] thermal cycler (MJ Research, Waltham, M.A., U.S.A.) and the samples subjected to the following conditions below. 105 µl of RNase-free water was added to each sample and stored at -20°C.

| | Step | Time (minutes) | Temperature (°C) |
|---|--------------------|----------------|------------------|
| 1 | Primer Annealing | 30 | 16 |
| 2 | Extension | 30 | 42 |
| 3 | Inactivation of RT | 5 | 85 |

2.4.3 miScript Reverse Transcription Kit

The miScript Reverse Transcription Kit (QIAGEN, Crawley, U.K.) comprises a unique reverse transcription mix that contains two enzymes: a poly(A) polymerase that polyadenylates mature miRNA molecules in the sample, and a traditional reverse transcriptase enzyme. The buffer contains random primers to reverse transcribe mRNA and oligo-dT primers to bind and transcribe miRNA products.

The following reaction was set up for each sample: 200 ng of DNase treated RNA was combined with 1 µl of Reverse Transcription Mix, 4 µl of 5X miScript Reverse Transcription Buffer and made up to 20 µl with RNase-free water in a 96 well plate. The reaction was run on the Multi Block System Satellite 0.2 Thermo Cooler (Thermo Fisher Scientific, U.K.) set with the following below. Following the reaction, 80 µl of RNase-free water was added to each sample and then stored at -20°C.

| Step | Time (minutes) | Temperature (°C) |
|------------------------|----------------|------------------|
| Reverse Transcription | 120 | 37 |
| Inactivation of RT Mix | 5 | 95 |

2.5 Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time PCR (qRT-PCR) was performed on cDNA template to determine the relative quantities of mRNA or miRNA in a sample. The reactions were set up using the methods described below in Thermo-Fast® 384-well PCR plate (Thermo Fisher Scientific, U.K.) and run on an ABI PRISM 7900HT PCR system. Triplicate reactions were set up for +RT cDNA samples and a duplicate for the -RT sample. Positive controls and water blanks were also included.

2.5.1 Universal ProbeLibrary

The Universal ProbeLibrary (UPL) System (Roche Applied Science, Indianapolis, USA) comprises 165 pre-validated, real-time PCR probes that are labelled with fluorescein (FAM) at the 5' end and a dark quencher dye at the 3' end. Primers were designed using the UPL assay design centre and ordered from Eurofins MWG Operon (Ebersberg, Germany). The amplicon for each primer pair encompassed a specific probe binding site and where possible, intron spanning primers were used. Details of primers and probes can be found in (Appendix, Table 8-7-1). Reaction master mixes were set up as follows;

| Reagent | Volume (µl) | Final Concentration |
|-----------------------------|-------------|---------------------|
| Absolute™ QPCR ROX MIX (2x) | 5.0 | 1X |
| Forward Primer (10 µM) | 0.4 | 400 nM |
| Reverse Primer (10 µM) | 0.4 | 400 nM |
| UPL Probe (10 µM) | 0.1 | 100 nM |
| Nuclease-Free Water | 2.1 | |

Two µl of cDNA prepared using either the ImPromII RT System or the miScript Reverse Transcription Kit (sections 2.4.1 and 2.4.3) was added to a 384-well PCR plate and the plate briefly centrifuged to collect the solution. 8 µl of the master mix was added to the appropriate well and mixed. The plate was sealed with an optical QPCR Absolute seal (ABgene Ltd, Epsom, U.K.), centrifuged to collect the reaction and run on the ABI PRISM 7900HT cyclor using SDS software with the conditions outlined below:

| Step | Time | Temperature (°C) | Cycles |
|-------------------------|------------|------------------|--------|
| Enzyme Activation | 15 minutes | 95 | 1 |
| Denaturation | 15 seconds | 95 | 40 |
| Annealing and Extension | 1 minute | 60 | |

2.5.2 TaqMan[®] qRT-PCR

TaqMan[®] pre-designed RT-PCR gene expression assays (Applied Biosystems, Warrington, U.K.) were used to measure relative quantities of PTK9 (Hs00702289_s1), Dicer-1 (Hs00229023_m1) and HMGA2 (Rn00583330_m1*) mRNA. A master mix was set up as detailed below, with 8 µl of this added to 2 µl of cDNA sample (section 2.4.1) in a 384-well optical plate. The reaction was run according to the conditions detailed in Section 2.5.1.

| Reagent | Volume (µl) |
|---|-------------|
| ABsolute [™] QPCR ROX MIX (2x) | 5 |
| TaqMan [®] Gene expression Assay | 0.5 |
| Nuclease-Free Water | 2.5 |

TaqMan[®] miRNA assays (Applied Biosystems, Warrington, U.K.) were used to measure relative quantities of mature miRNA, details of which are in (Appendix; Table 8-7-2). 1.5 µl of cDNA, produced using the TaqMan[®] MicroRNA Reverse Transcription Kit (section 2.4.2), was added to a 384-well optical plate with 8.5 µl of miRNA Taqman[®] master mix, detailed below. This reaction was run according to the cycling conditions in Section 2.5.1.

| Reagent | Volume (µl) |
|--|-------------|
| ABsolute [™] QPCR ROX MIX (2x) | 5.0 |
| TaqMan [®] miRNA expression Assay | 0.5 |
| Nuclease-Free Water | 3.0 |

2.5.3 *miScript qRT-PCR System*

Detection of mature miRNA was performed using the miScript SYBR Green PCR Kit (QIAGEN, Crawley, U.K.) and cDNA template generated using the miScript Reverse Transcription Kit (Section 2.4.3). 10X miScript Primer Assays (Appendix; Table 8-7-3) were reconstituted with 550 μ l of TE (pH 8.0) and stored at -20°C. A reaction mix was set up, as detailed below and 8 μ l of this was added to cDNA, prepared as in Section 2.4.3, in a 384 well plate.

| Reagent | Volume (μ l) |
|---|-------------------|
| QuantiTect SYBR Green PCR Master Mix (2X) | 10 |
| 10X miScript Universal Primer | 2 |
| 10X miScript Primer Assay | 2 |
| Nuclease-Free Water | 4 |

The reaction was subjected to the conditions detailed below on an ABI PRISM 7900HT PCR cycler. A melt curve was performed immediately after the cycling conditions to verify specificity and identity of PCR products; data was collected during the 60°C-95°C ramp phase. A single peak was observed for each primer assay, confirming a single and unique PCR product.

| Step | Time | Temperature (°C) | Cycles |
|-------------------|------------|------------------|--------|
| Enzyme Activation | 15 minutes | 95 | 1 |
| Denature | 15 seconds | 95 | 40 |
| Annealing | 30 seconds | 55 | |
| Extension | 30 seconds | 70 | |
| | 15 seconds | 95 | 1 |
| Melt curve | 15 seconds | 60 | |
| | 15 seconds | 95 (slow ramp) | |

2.5.4 Analysis of qRT-PCR Results

The extension phase of the PCR releases the fluorescence from the respective qRT-PCR probe, which is measured by the ABI PRISM 7900HT thermo cycler. A threshold of fluorescence is set during the exponential phase of amplification. The cycle number at which the fluorescence signal breaches this set threshold is termed the cycle threshold (C_t).

qRT-PCR results were analysed using the relative quantification method of comparative C_t . This method assumes the efficiencies of each assays under comparison to be approximately equal. Efficiencies were calculated using serial dilutions of cDNA product and the equation:

$$\text{Efficiency (\%)} = (10^{-1/\text{slope}}) \times 100$$

The genes *GAPDH* and *B-Actin*, and small nucleolar RNA 48 (*RNU48*) were used as housekeeping genes and assays for each were run on all plates. These act as a normalising control for RNA concentration and small pipetting discrepancies. They had each been tested for consistent expression and reproducibility in appropriate samples. Housekeeping gene expression was used to calculate the ΔC_t for the given gene of interest as follows;

$$\Delta C_t = C_{t(\text{sample})} - C_{t(\text{Housekeeper})}$$

All experiments were compared to a reference control (e.g. Non transfected cells) and the $\Delta\Delta C_t$ calculated as follows;

$$\Delta\Delta C_t = \Delta C_{t(\text{sample})} - \Delta C_{t(\text{reference})}$$

The fold change in mRNA or miRNA was calculated as follows;

$$\text{Fold Change} = 2^{-\Delta\Delta C_t}$$

2.6 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed on a subset of the North Glasgow Monitoring Trends and Determinants in Cardiovascular (MONICA) normotensive cohort to facilitate sequencing of the 3'UTR region of the *CYP11B1* gene.

2.6.1 PCR of *CYP11B1* 3'UTR

Selected DNA samples previously extracted from patients of the MONICA study were quantified using the Nanodrop (section 2.2.4) and diluted to 20 ng/μl. The following reaction used primers, detailed in Table 2-1, and the Expand Hi-Fidelity PCR System (Roche Applied Science, Indianapolis, USA);

| Reagent | Volume per Reaction (μl) |
|---|--------------------------|
| Expand High Fidelity Buffer 10x with (MgCl ₂) | 2.5 |
| dNTP Mix | 2.0 |
| Forward Primer (10 μM) | 1.0 |
| Reverse Primer (10 μM) | 1.0 |
| Expand High-Fidelity Enzyme Mix | 1.0 |
| Nucleases-Free Water | 12.5 |
| DNA | 5.0 |
| Total | 20 |

Table 2-1. Primers Used to Amplify *CYP11B1* 3'UTR.

| Name | Sequence (5'-3') | Direction | T _m (°C) |
|--------------|----------------------|------------|---------------------|
| B1 ex9 seq1F | CAGGTGGAGACACTAACCCA | Sense | 59.3 |
| B1 ex9 R1 | ATGCTCTGCCCCTGCAGCTT | Anti-Sense | 61.4 |

Reactions were set up in a 96-well plate and subjected to the following conditions on a 96-well Dyad Disciple™ sample block powered by the PTC-0221 thermal cycler (MJ Research, Waltham, M.A, USA).

| Time (seconds) | Temperature (°C) | Cycles |
|------------------------|------------------|--------|
| 120 | 94 | 1 |
| 15 | 94 | 10 |
| 30 | 66 | |
| 90 | 72 | |
| 15 | 94 | |
| 30 | 66 | 20 |
| 90 + 5 seconds / cycle | 72 | |
| 420 | 72 | 1 |

The PCR product (2,225 base pairs) was verified by agarose gel electrophoresis (section 2.6.2) prior to sequencing (section 2.7).

2.6.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for analysing PCR fragments or restriction endonuclease reactions. A 0.7% agarose gel was prepared by combining 0.7% (w/v) Ultrapure agarose (Invitrogen, Paisley, U.K.) with 100 ml 1x Tris/Borate/EDTA (TBE) (10 mM Tris, 10 mM boric acid, 10 mM EDTA, pH 8.3) and heating in a microwave oven for approximately 80 seconds. Ethidium bromide, 1 µl (10 mg/ml) (Sigma-Aldrich, Poole, U.K.) was added to the liquid gel in a fume hood; this intercalates with the nucleic acids and fluoresces under UV light. The gel was poured into a gel mould with a Teflon comb and allowed to set for 30 minutes, then submerged in 1 X TBE buffer in an electrophoresis tank and the comb removed.

The samples were combined with loading dye (0.02% bromophenol blue, 0.02% xylene cyanol and 2.5% glycerol) and carefully loaded into the cast wells. 5 µl of 1 kb DNA size ladder (Promega, Madison, W.I, USA) was run to allow the resolution of molecular size. The gel was resolved at a constant voltage of 80 V for 60 minutes. DNA fragments were visualised and captured using UV light on a molecular Imager ChemiDoc™ XRS+ Imaging system (Bio-Rad Laboratories, Hemel Hempstead, U.K.) and Multi-Analyst software v 1.1 (Bio-Rad).

2.7 DNA Sequencing Reaction

2.7.1 PCR Purification

The AMPure purification method (Agencourt, USA) was used to purify PCR products from PCR reagents. For PCR reactions with a final volume of 20 µl, 36 µl of resuspended, room temperature AMPure reagent was added to each well, the plate sealed, vortexed to mix and left to sit at room temperature for 5 minutes. The PCR products, at least 100 base pairs, bind to the magnetic beads. The plate was then placed on an SPRI (solid phase reversible immobilization) magnet for 5 minutes to draw the beads/PCR product from the liquid phase, which was subsequently cleared from the plate by inversion. The beads/PCR product was washed by two 200 µl volumes of 70%. Following the second wash, the plate was inverted on a tissue and centrifuged at 76 x g for 30 seconds, then removed from the magnet and left to dry for 10 minutes.

The PCR product was eluted from the beads by adding 40 µl of nuclease-free water, vortexing and placing the plate back on the magnet. This cleaned PCR product was then transferred to a new plate for use in the sequencing reaction (section 2.7.2) or stored at -20°C.

2.7.2 Sequencing Reaction

DNA sequencing was performed using 10 µl of purified PCR product or 200 ng of plasmid DNA and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, U.K.). The following components were set up in a 96-well plate:

| Reagent | Volume per Reaction (µl) |
|---|--------------------------|
| ABI PRISM BigDye v3.1 Sequencing Buffer | 4.0 |
| ABI PRISM BigDye Terminator v3.1 Ready Reaction Mix | 1.0 |
| Primer (3.2 µM) | 1.0 |
| Plasmid DNA or PCR product | Variable |
| Nuclease-Free Water | Up to 20 µl |

Details of sequencing primers can be found in Table 6-1. The sequencing reaction was subjected to the following conditions on a 96-well heating block.

| Time (minutes) | Temperature (°C) | Cycles |
|----------------|------------------|--------|
| 45 | 96 | 25 |
| 240 | 60 | |

2.7.3 Sequencing Reaction Purification

The CleanSEQ purification method (Agencourt, USA) was used to remove reagents from the sequencing product. 10 µl of resuspended, room temperature CleanSEQ and 62 µl of freshly prepared 85% ethanol were each added to a 20 µl sequencing reaction. The plate was resealed, vortexed and briefly centrifuged, then placed on a SPRI magnet for 5 minutes. The liquid phase was cleared from the wells by inversion then washed in 150 µl of 85% ethanol for 30 seconds. The plate was inverted and centrifuged at 76 x g for 30 seconds to remove ethanol. The plate, off the magnet, was then left to air dry at room temperature for 10 minutes.

To elute the sequencing product from the beads, 40 µl of nuclease-free water was added to each well, the plate sealed, vortexed and centrifuged then placed onto the SPRI magnet to remove beads from the purified sequencing reaction.

2.7.4 Automated Cycle Sequencing

To analyse sequencing products, 20 µl of the purified sequencing reaction was transferred to a 96-well bar-coded plate. Sequencing was analysed using the Applied Biosystems 3730 DNA Analyzer and SeqScape v 2.1.1 software.

2.8 pEZX - 3'UTR Reporter Construct

Reporter constructs were purchased from LabOmics (Nivelles, Belgium) to determine miRNA binding to the 3'UTR. Each construct contained a pEZX-reporter backbone, which comprises a renilla reporter gene coupled to a SV40 (Simian virus 40) viral promoter, a firefly experimental gene coupled to a CMV (Cytomegalovirus) promoter with a multiple cloning site located at the 3' end of the gene, and a Kanamycin resistant gene for antibiotic selection. Constructs containing the 3'UTR of *CYP11B1* (pEZX-B1), the 3'UTR of *CYP11B2* (pEZX-B2) or a negative control empty vector (pEZX-Con) were purchased (Figure 2-1). The constructs were transformed and grown as detailed below.

2.8.1 Transformation of Competent Cells

One Shot[®] TOP10 *E.coli* competent cells (Invitrogen, Paisley, U.K.) were transformed according to the manufacturer's protocol. Briefly, 1 vial (50 µl) of cells was thawed on ice for each reaction, and then briefly centrifuged to collect cells. 1 µl of DNA (approximately 50 pg) was added and the tubes were gently flicked to mix. The cells were incubated on ice for 30 minutes, then heat-shocked by placing vials in a 42°C water bath for 30 seconds. The cells were cooled by placing on ice for 2 minutes. 200 µl of room temperature SOC (super optimal broth catabolite repression) medium was added to each vial before shaking at 225 rpm, 37°C for 1 hr in an Innova[®] 44 incubator shaker (New Brunswick Scientific, St. Albans, U.K.). 200 µl of the transformation reaction was plated onto LB (Luria Broth) Agar plates with kanamycin (50 mg/ml) and incubated overnight at 37°C.

Plates were inspected the following day. Single colonies were picked from the plate using sterile pipette tips and placed into individual 15 mL tubes with 2 ml of LB, supplemented with Kanamycin (50 mg/ml). These starter cultures were shaken overnight at 37°C at 225 rpm.

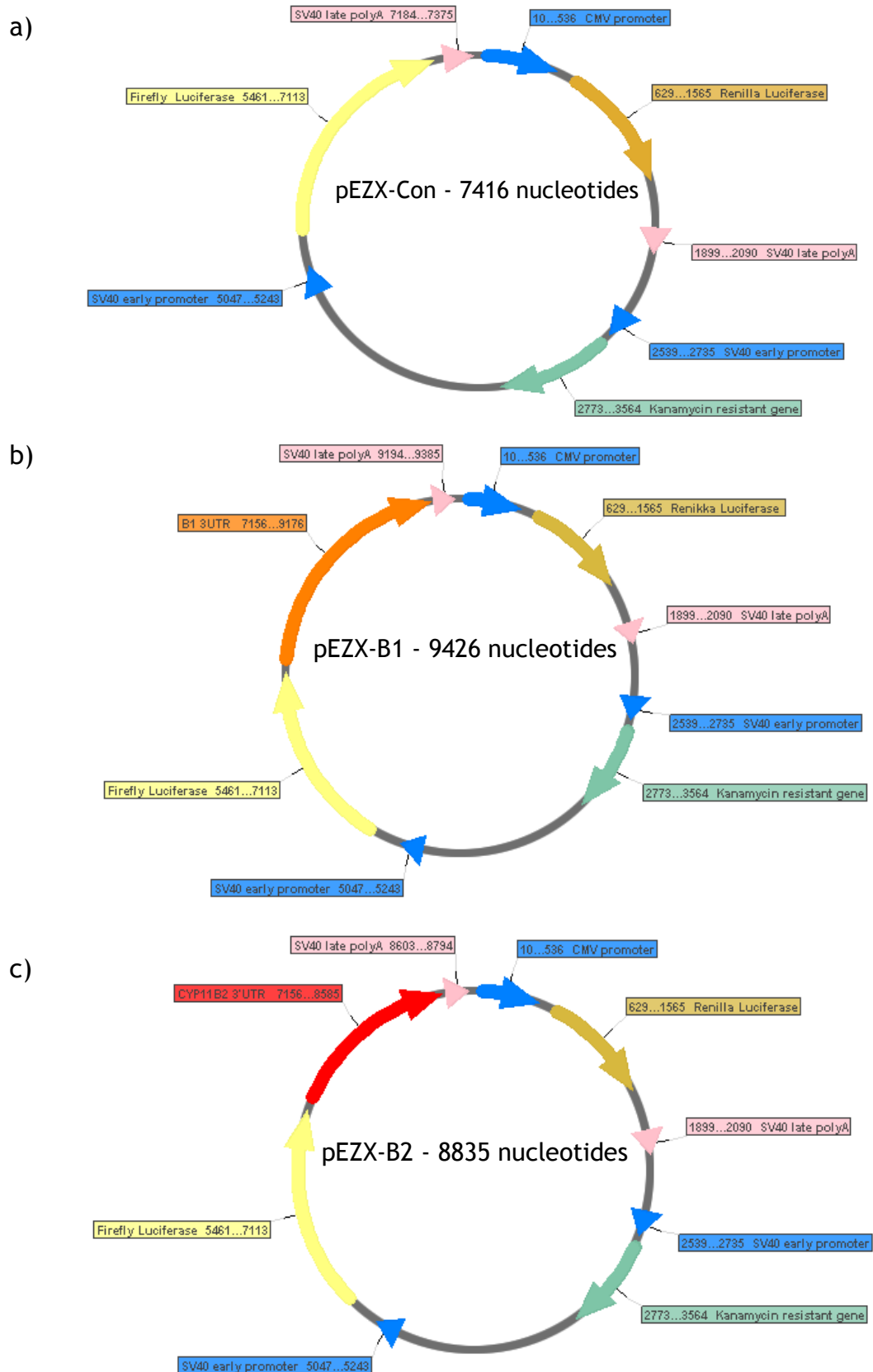


Figure 2-1. pEZX Reporter Constructs.

Outline of the pEZX vector map a) pEZX-control, b) pEZX-B1, c) pEZX-B2. Schematic generated using SerialCloner v2.0 software.

2.8.2 Small Scale DNA Purification

The QIAprep Spin Miniprep Kit (QIAGEN, Crawley, U.K.) was used to purify plasmid DNA from overnight incubations. 1 ml of the incubation was transferred into a 1.5 ml tube, which was centrifuged at 10,000 x g for 10 minutes to pellet the cells. The supernatant was discarded and the cells lysed by resuspending them in 250 µl of Buffer P1. The DNA was denatured by adding 250 µl Buffer P2 and mixing by inversion. This reaction was neutralised by adding 350 µl of Buffer N3 and immediately mixing by inversion. The samples were centrifuged for 10 minutes at 13,000 x g in a table top centrifuge. The supernatants were transferred into a QIAprep spin column, which was centrifuged at 13,000 x g for 1 minute to load DNA on to the silica membrane. The flow through was discarded. The column was washed by adding 500 µl of Buffer PB and centrifuging at 13,000 x g for 1 minute. Another wash with 750 µl of Buffer PE was performed, after which the column was centrifuged at top speed for 1 minute to ensure there was no residual buffer remaining. The column was transferred to a new 1.5 mL tube and the DNA eluted by adding 50 µl of nuclease-free water, standing at room temperature for 1 minute then centrifuging at 13,000 x g for 1 minute. Plasmid DNA was quantified by Nanodrop (Section 2.2.4) and stored at -20°C until required.

2.8.3 Large Scale DNA Purification

Large bacterial culture preparations were set up using 500 µl of the starter culture (Section 2.8.2) added to 400 ml of LB and supplemented with kanamycin. The culture was shaken at 37°C, 225 rpm overnight.

The QIAGEN® Plasmid Purification Kit (Maxi) was used to purify plasmid DNA from *e. coli* cells on a large scale. Following the manufacturer's protocol, the cells were collected in an ultra centrifuge at 6,000 x g for 15 minutes at 4°C, then the cell pellet was resuspended in 10 ml of Buffer P1. To these lysed cells, 10 ml of Buffer P2 was added, mixed by shaking, then 10 ml of chilled Buffer P3 was added and mixed by inversion. The solution was centrifuged at 20,000 x g for 30 minutes at 4°C to remove cell debris. The supernatant containing the DNA was removed and applied to a QIAGEN-tip 100 and the sample allowed to run through

the resin by gravity. The QIAGEN-tip was washed twice with 10 ml of Buffer QC. The DNA was eluted with 5 ml of Buffer QF and precipitated by adding 3.5 ml of isopropanol and centrifuged at 15,000 x g for 30 minutes at 4°C. The pelleted DNA was washed with 2 ml of 70% ethanol, which was placed into multiple 1.5 ml tubes and centrifuged at maximum speed for 10 minutes. The supernatant was discarded and the pellet air dried. Finally, the DNA was eluted by resuspending and combining the DNA in nuclease-free water. The DNA was quantified (Section 2.2.4), used in transfection and stored at -20°C.

2.8.4 Restriction Endonuclease Digestion

Restriction digests were performed using enzymes shown in Table 2-2 to establish the molecular size of the plasmid and insert. Approximately 500 ng of plasmid DNA was combined with 1 µl of enzyme (1 unit), 2 µl of buffer, specific to each enzyme (Table 2-2), 0.2 µl of BSA (except with *HindIII*) and nuclease-free water, up to a final volume of 20 µl in a 96-well plate. Control reactions included those omitting DNA or enzyme. The plate was placed at 37°C for 2 hours. The product was analysed by agarose gel electrophoresis (Section 2.6.2)

Table 2-2. Endonuclease Enzymes Used for Plasmid Digestion.

| Enzyme | Brand | Buffer |
|----------------|-------------------------|--------|
| <i>HindIII</i> | New England Biosciences | 2 |
| <i>SacI</i> | New England Biosciences | 4 |
| <i>BamHI</i> | New England Biosciences | 3 |
| <i>StuI</i> | Promega | B |

2.9 Transient Cell Line Transfection

2.9.1 Preparation of Small Molecules

Specific miRNA mimics (Pre-miR™) and miRNA antagonists (Anti-miR™) were purchased from Applied Bioscience (Warrington, U.K.) to test the effects of particular miRNAs on *CYP11B1* and *CYP11B2* mRNA *in vitro*. Scrambled control molecules for each Pre-miR and Anti-miR were also purchased. Details of the molecules used can be found in Table 5-1. Each Pre-miR™ or Anti-miR™ was resuspended to 6.25 µmoles by adding 800 µl of RNase-free water, vortexing to mix and collecting the contents by centrifugation. Molecules were stored at -20°C.

Two siRNAs designed to target human *Dicer-1* mRNA (s23755 and s23756; Figure 8-7-1) were purchased from Applied Biosystems (Warrington, U.K.) and used to determine the effect of global down regulation of miRNAs. A positive control *GAPDH*-targeted siRNA and a scrambled negative control siRNA were also purchased. The siRNA molecules were reconstituted to a final concentration of 10 µM and stored at -20°C

2.9.2 Small Molecule Transient Transfection

Small molecules (Pre-miR™, Anti-miR™ or siRNAs) were transfected into H295R cells in 6-well plates. Reactions were performed in triplicate. Following the transfection agent manufacturer's protocol, cells were trypsinised as in section 2.1.3 and counted as in section 2.1.4. Cells were diluted to a concentration of 2×10^5 cells/ml using normal growth medium and the suspension placed in the 37°C incubator until required.

A transfection reagent master mix was prepared by adding 9 µl of siPORT™ NeoFX transfection (Applied Bioscience, Warrington, U.K.) to 291 µl of OptiMEM® I reduced serum medium (Invitrogen, Paisley, U.K.) per reaction plus a 10% excess in a 30 mL tube and kept in the dark for 10 minutes.

Small molecules were prepared for transfection by diluting them in OptiMEM® medium as detailed in Table 2-3.

Table 2-3. Constituents of Transfection Reaction.

| Small Molecule | Stock Concentration (μmoles) | Volume (μl) | Final concentration (nM) | Volume of OptiMEM (μl) |
|----------------|------------------------------|-------------|--------------------------|------------------------|
| siRNA | 10.00 | 9 | 30 | 291 |
| Pre-miR™ | 6.25 | 24 | 50 | 274 |
| Anti-miR™ | 6.25 | 24 | 50 | 274 |

The transfection agent and small molecules were combined by adding 300 μl of the siPORT™ NeoFX/OptiMEM to each transfectant, mixing and incubating for 10 minutes at room temperature.

Following the incubation, 600 μl of the transfection complex was then added to the cell culture plate. The cells were then removed from the incubator, resuspended and 2.4 ml of the cells added to each well to a final cell density of 4.8×10^5 cell/well. The plate was rocked to mix the reagents and then placed in the incubator.

After 24 hours the medium on the cells was exchanged for 3 ml of normal growth medium. After a further 24 hours the medium was removed and stored at -20°C for subsequent steroid analysis (Section 2.11) The cells were lysed for RNA (Section 2.2.1) or protein (Section 2.12) isolation.

2.9.3 Reporter Construct Transient Transfection

The pEZX-reporter construct (section 2.6) was transfected into H295R or HeLa cells in quadruplicate on a 24-well plate. The transfection was set up in a similar manner as detailed in Section 2.9.2, except that the volumes of reagents used were reduced. For a 24-well plate, 1.5 μl of siPORT™ NeoFX and 48.5 μl of OptiMEM® I were combined and incubated for 10 minutes. The pEZX-construct (100 ng/μl) was combined with OptiMEM® medium to a final concentration of 50 μl (Table 2-4).

Table 2-4. Constituents of Transfection Reaction for pEZX-Reporter Construct.

| | Stock Concentration (ng/μl) | Volume (μl) | Final Concentration (nM) | OptiMEM Volume (μl) |
|----------------|-----------------------------|-------------|--------------------------|---------------------|
| pEZX construct | 100 | 1 | 100 | 49 |

50 µl of the NeoFX/OptiMEM[®] mix was added to each transfectant, mixed and then incubated for 10 minutes at room temperature. Following the incubation, 100 µl of the transfection complex was added to each well of the cell culture plate. The cells were removed from the incubator, mixed to resuspend and 400 µl added to each well, to a final cell density of 8×10^4 cells/well. The plate was rocked to mix the reagents and then placed in the 37°C incubator.

The medium was changed after 24 hours. After a further 24 hours the medium was removed and discarded, and the cells were lysed with 100 µl of 1X Passive Lysis Buffer (1X PLB) (section 2.10) and stored at -80°C, for subsequent luciferase analysis.

2.9.4 Co-transfection

The pEZX-reporter construct was co-transfected with Pre-miR[™] or Anti-miR[™] molecules to directly investigate the effects of miRNAs on the 3'UTR of the *CYP11B1* or *CYP11B2* genes. This was performed using HeLa cells in 24-well plates, in a similar manner to that described above. This pEZX-construct (100 ng) was added to Pre-miR[™] or Anti-miR[™] molecules (final concentration 50 nM) and made to a final volume of 50 µl of OptiMEM[®] medium.

2.10 Dual Reporter Luciferase Assay

Renilla and Firefly luciferase activity was measured in cells, lysed in 1X passive lysis buffer, using the Dual Luciferase Reporter Assay system (Promega, Madison, WI, USA) on a Lumat LB 9507 tube luminometer (Berthold Technologies, Harpenden, U.K.). All reagents were thawed at room temperature and then prepared by reconstituting the lyophilised luciferase assay substrate in 10 ml of luciferase assay buffer II (LAR II) and adding 400 µl of Stop and Glo Substrate to 10 ml of Stop and Glo Buffer. The luminometer was prepared by priming line 1 with the Luciferase assay reagent and line 2 with Stop and Glow reagent. 10 µl of lysates were added to round bottomed tubes, in duplicate, and loaded into the luminometer. The Lumat 9507 was set to automatically inject 50 µl of LAR II reagent, pause for 2 seconds then measure luciferase for 10 seconds. This was immediately followed by an injection of 50 µl Stop and Glo reagent, a further 2

second pause and then a luciferase measurement over 10 seconds. Firefly luciferase activity measured in relative light units (RLU) was divided by renilla luciferase activity (RLU) to normalise for transfection efficiency and the mean taken of the duplicate ratios.

2.11 Steroid Quantification

Cell media was stored at -20°C until steroids could be measured using Liquid Chromatography Tandem Mass spectrophotometry (LC:MS/MS) by Miss Mary Ingram. Steroids were extracted from the media using Chem Elute cartridges (Varian, CA, USA) and eluted with dichloromethane. The sample was then evaporated to dryness under nitrogen and reconstituted in 60 µl acetonitrile. A 20 µl aliquot was injected into a Polaris 5 micron, 150 mm x 2 mm C-18-A reversed phase HPLC column. Identification and quantification of steroid products was achieved by tandem mass spectrometry using a Varian 1200L mass spectrometer with a triple quadrupole detector.

2.12 Determination of Protein Concentration

Total protein concentration in cells lysed in 1 X Reporter Lysis Buffer (1X RLB) was measured using a bicinchoninic acid (BCA) assay kit (PIERCE, Perbio Science, Northumberland, U.K.) following the manufacturer's protocol. A BCA working reagent (WR) was prepared by mixing BCA Reagent A with BCA Reagent B at a ratio of 50:1 (A:B). Protein standards were prepared using the albumin standard included in the kit and 1X RLB to cover a range of 25 µg/ml - 2000 µg/ml, 1X RLB was used as the blank for calibration. In duplicate, 25 µl of protein standards or unknown sample was added to a 96-well flat bottom plate, to which 200 µl of the working reagent was added. The plate was incubated at 37°C for 30 minutes then cooled prior to measuring the samples' absorbance at 560 nm, using a Wallac Victor² plate reader (Wallac, Turku, Finland). The blank absorbance reading (1X RLB) was subtracted from each sample or standard reading. A standard curve was plotted and the concentration of the samples extrapolated.

2.13 Statistical Analysis

Statistical analysis was performed using Prism 4.0 Graph Pad software. All results are expressed as mean \pm SEM (standard error of the mean). *In vitro* experiments were performed in at least three technical replicates, at three independent biological times (unless otherwise stated). *In vitro* results were analysed by either an unpaired Student's t-test or one-sample t-test as stated. Analysis of results for multiple groups was performed by one-way analysis of variance (ANOVA) and Bonferroni's post-hoc test, comparing all results to a designated control group or all sets of data as required.

Confidence intervals of 95% were used, with a P-value of < 0.05 therefore being considered significant.

3 Investigating a Role for miRNA Regulation in Adrenal Cells

3.1 Introduction

miRNA molecules are novel regulators of mRNA levels and protein expression (Section 1.5 and 1.6). The RNase III, Dicer, is essential for miRNA maturation and its deletion in the SF-1 positive cells of mouse embryos prevents adrenal formation (Huang and Yao, 2010). Therefore, miRNAs are crucial to the embryonic development of the adrenal gland. However, their influence on corticosteroidogenesis has not been established. This study uses Dicer-targeted siRNAs (Section 1.5.1) and 3'UTR reporter constructs, each transfected into an adrenocortical cell line, to determine which enzymes in the corticosteroidogenic pathway are subject to miRNA-regulation.

In order to understand whether miRNAs regulate genes in the adrenal gland a suitable *in vitro* model is required. The most widely used cell model of adrenal steroidogenesis is the NCI-H295 cell line. It was established from an adrenocortical carcinoma mass that was surgically removed from a 48-year-old black female. Initial characterisation of this cell line concluded that it continued to synthesise and secrete most adrenal steroids (Gazdar *et al.*, 1990) but cells had to be maintained in suspension. Subsequent manipulations of growth conditions resulted in an adherent cell line, H295R, which retains steroid producing capabilities, largely due to the use of the bovine-derived serum substitute, Ultrosor G. A modified strain of this cell line was also derived using Nu-Serum in place of Ultrosor G, which overcomes the limited availability which can be an issue with Ultrosor G. This cell line is available from the American Type Culture Collection (ATCC) (Rainey *et al.*, 1994). For studies in this thesis, H295R cells maintained in Ultrosor G were used. Unlike the spatial separation of corticosteroidogenesis that occurs in discrete zones of the adrenal gland, the H295R cells co-express enzymes for the biosynthesis of mineralocorticoids, glucocorticoids and androgens and therefore secrete these various steroids. The cells are responsive to AngII and K^+ and express the AT1R (Bird *et al.*, 1993). They do not express the ACTH receptor and are therefore unresponsive to ACTH stimulation (Mountjoy *et al.*, 1994). The cells are commonly used for investigating various aspects of steroid production including transcription factor, inhibitor and mutational studies (Lehoux *et al.*, 2001; Ye *et al.*, 2009).

The expression of miR-21 has been assessed in H295R cells, where it was shown to increase in response to AngII stimulation and to be associated with raised aldosterone production (Romero *et al.*, 2008). However, the molecular mechanism of miR-21 action was not investigated and this remains the only report of miRNA modulation of steroid production to date. In order to test whether miRNAs exert control over the corticosteroidogenic pathway, it would be highly desirable to measure the levels of relative mRNA and steroid in order to gain the fullest possible picture of their effects.

3.2 Aims

The aim of this study was to determine whether miRNA-mediated regulation is an important factor in the regulation of steroidogenic enzyme production and of corticosteroid biosynthesis in adrenal cells. Additionally, this study examined whether miRNA regulation of the *CYP11B1* and *CYP11B2* genes occurs at their respective 3'UTRs and whether this is modulated by AngII stimulation.

3.3 Methods

3.3.1 *Transfection of H295R Cells with siRNA molecules*

H295R cells were transfected in 6-well plates as described in section 2.9. Six replicate wells per condition were set up on three independent occasions. The siRNA molecules used and the preparation methods are detailed in section 2.9.1. The medium was replaced on the cells after 24 hours and, after 48 hours, the medium was removed for steroid analysis by LC:MS/MS (Section 2.11). Cell lysates were prepared for protein quantification from three replicates (Section 2.12). Steroid concentrations were normalised per mg of protein and then expressed as fold-change compared to control siRNA transfected cells. The final three replicates were used for qRT-PCR analysis as described in section 2.4 and 2.5. Data were analysed using the $\Delta\Delta C_t$ method (Section 2.5.4) with β -actin as a housekeeping gene, and results expressed relative to the control siRNA transfected cells.

3.3.2 *3'UTR Reporter Construct Plasmids*

The pEZX-control, pEZX-B1 and pEZX-B2 3'UTR reporter construct plasmids (Figure 2-1) were prepared as described in section 2.8.3. For verification, each construct was digested with several restriction endonuclease enzymes (Section 2.8.4) and resolved by agarose gel electrophoresis (Section 2.6.2). Also, the 3'UTR insert and a small region covering the multiple cloning site of each plasmid was sequenced, as described in section 2.7.

3.3.3 *Investigation of 3'UTR Activity In Vitro*

H295R cells were transfected with the pEZX reporter constructs in 24-well plates as described in section 2.9.3. Cell medium was replaced at 24 hours with normal growth medium. Further, at 1, 6 and 24 hours prior to cell lysate preparation, medium was removed and replaced with normal growth medium (control, non-stimulated cells) or normal growth medium supplemented with AngII (10 nM), (Section 2.1.5). 48 Hours post-transfection, cell lysates were prepared and their luciferase activities measured DRLA (Section 2.10). As controls non-transfected

cells were used as well as cells transfected with construct containing only virally-driven firefly (pGL4.13) or renilla reporter gene (pGL4.73, Promega Madison, WI, USA). These were prepared as described in section 2.8.3. and acted as transfection controls and as controls for background luminescence, and also determined whether firefly luminescence was fully quenched by the Stop and Glo reagent of the DLRA. Normalisation for transfection efficiency was achieved by dividing firefly luciferase luminescence by renilla luciferase luminescence (both expressed as RLU). The firefly to renilla ratio from AngII-stimulated cells was expressed as a percentage of that from non-stimulated time-matched control transfected cells.

3.3.4 Statistical Analysis

Statistical difference in mRNA or steroid levels in siRNA experiments was assessed by a one-way analysis of variance (ANOVA) test and by Bonferroni's post-hoc test, comparing the Dicer1 siRNA results for each with that from control siRNA-transfected cells. Reporter construct results were analysed by one-way ANOVA and Bonferroni's post-hoc tests, comparing all time-points.

For all analyses, confidence intervals of 95% were used and $P < 0.05$ was required for statistical significance. Data are expressed as the mean \pm standard error of the mean (SEM).

3.4 Results

3.4.1 Confirmation of siRNA Transfection

The suitability of the transfection conditions for siRNA delivery into H295R cells was assessed using a positive control siRNA targeted against *GAPDH* mRNA. Following the transfection, RNA was isolated and the levels of *GAPDH* mRNA measured by qRT-PCR. *GAPDH*-specific siRNA significantly reduced *GAPDH* mRNA levels to 0.46 ± 0.03 to that of the control transfected cell ($p < 0.001$) (Figure 3-1).

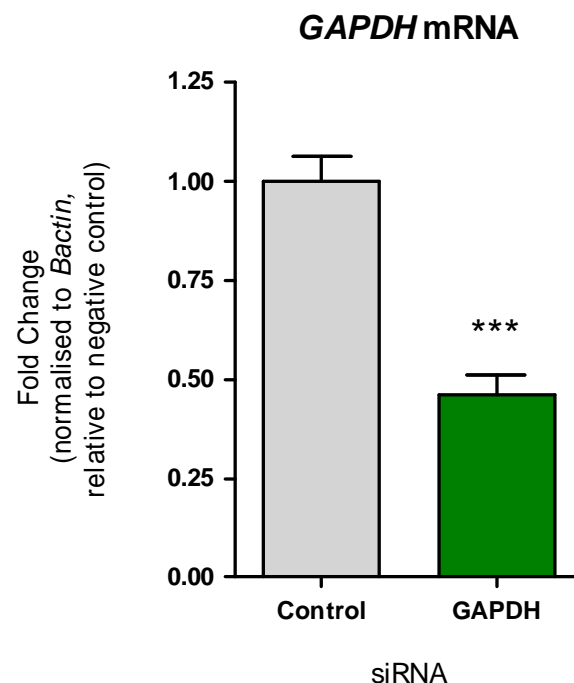


Figure 3-1. Assessment of the *GAPDH*-targeted siRNA on *GAPDH* mRNA in H295R Cells.

H295R cells were transfected with scrambled negative control siRNA or a *GAPDH*-specific siRNA (final concentration 30 nM). *GAPDH* mRNA was analysed 48 hours post-transfection by qRT-PCR. Cycle threshold values were normalised to *β-actin* mRNA and expressed relative to control cell values. The mean of three independent biological experiments performed in triplicate, error bars represent SEM. *** $p < 0.001$ compared to control.

3.4.2 siRNA-mediated Dicer-knockdown in H295R cells

Following transfection of the H295R cells with Dicer1-specific siRNAs, targeting was validated by measuring *Dicer1* mRNA levels using qRT-PCR (Section 3.4.1). Dicer1 A siRNA significantly decreased *Dicer1* mRNA to 0.56 ± 0.07 fold ($p < 0.001$) and Dicer1 B siRNA decreased *Dicer1* mRNA to 0.50 ± 0.06 fold ($p < 0.001$), compared to control siRNA transfected cells (1.01 ± 0.05 ; Figure 3-2).

This depletion of *Dicer1* mRNA had no significant effect on *StAR*, *3 β -HSD11* or *HSD11B2* mRNA abundance (Figure 3-3 A,C and F). CYP11A1 mRNA abundance increased from control cell levels (1.03 ± 0.10) following transfection of Dicer1 A siRNA (1.41 ± 0.09 ; $p < 0.05$) and of Dicer1 B siRNA (1.42 ± 0.10 ; $p < 0.05$; Figure 3-3B). Dicer1 A siRNA significantly increased *CYP21A1* mRNA abundance from 1.01 ± 0.05 in control cells to 2.40 ± 0.34 fold ($p < 0.01$). However, the change in *CYP21A1* mRNA with Dicer1 B siRNA (1.72 ± 0.30) did not reach statistical significance relative to control (Figure 3-3D). The results for *CYP17A1* mRNA showed a similar pattern, with Dicer1 A siRNA significantly increasing abundance relative to control (1.73 ± 0.22 vs 1.02 ± 0.07 ; $p < 0.01$) but Dicer1 B siRNA (1.37 ± 0.07) not achieving statistical significance (Figure 3-3E).

Reducing *Dicer1* mRNA led to a significant increase in the relative level of *CYP11B1* mRNA present in cells transfected with Dicer1 A siRNA from 1.00 ± 0.04 to 1.75 ± 0.24 ($p < 0.05$) (Figure 3-4A). There was no significant change in cells transfected with Dicer1 B siRNA (1.39 ± 0.23). In addition, there was a significant increase in *CYP11B2* mRNA abundance with Dicer1 A siRNA from 1.00 ± 0.02 to 2.12 ± 0.41 ($p < 0.01$). However, Dicer1 B siRNA (1.40 ± 0.09) did not result in a statistically-significant increase (Figure 3-4B).

24-Hour steroid secretion was measured in the media of transfected cells by LC:MS/MS. Figure 3-5 shows the results for steroids synthesised on the aldosterone arm of the corticosteroid pathway. Firstly, deoxycorticosterone (DOC) levels (panel A) were significantly increased relative to control cell (1.00 ± 0.02) for both Dicer-targeting siRNAs (Dicer1 A, 1.23 ± 0.01 , $p < 0.05$ relative to control; Dicer1 B siRNA, 1.53 ± 0.09 , $p < 0.001$ relative to control). There was a significant change in corticosterone production between cells transfected with control siRNA or with Dicer1 siRNA A (1.00 ± 0.03 and 1.10 ± 0.06 , respectively;

Figure 3-5B). In contrast, corticosterone secretion was significantly increased in cells transfected with Dicer1 B siRNA 1.32 ± 0.13 fold ($p < 0.05$ relative to control). A similar pattern was observed with 18-OH-corticosterone production, with no change to control levels following Dicer1 A siRNA transfection (1.00 ± 0.01 and 1.17 ± 0.06 , respectively) and a slight but significant increase following Dicer1 B siRNA transfection to 1.28 ± 0.10 ($p < 0.05$ relative to control) (Figure 3-5C). Control aldosterone levels (1.00 ± 0.06) were similar following Dicer1 A siRNA transfection (1.06 ± 0.06) but significantly increased in Dicer1 B siRNA transfected cells (1.47 ± 0.11 fold; $p < 0.01$; Figure 3-5D).

Cortisol-related steroids are shown in Figure 3-6. There was no significant change in 11-deoxycortisol secretion (control cells 1.00 ± 0.01 ; Dicer1 A siRNA cells 1.14 ± 0.07 ; Dicer1 B siRNA cells 1.26 ± 0.12 ; Figure 3-6A). There was no significant difference in cortisol secretion following in Dicer1 A siRNA transfection (control cells 1.00 ± 0.01 ; Dicer1 A siRNA cells 1.07 ± 0.08) but cells transfected with Dicer1 B siRNA had significantly increased cortisol (1.33 ± 0.11 fold, $p < 0.05$; Figure 3-6B). Secretion of the cortisol metabolite cortisone did not change significantly in cells transfected with either Dicer siRNA molecule, relative to control (Figure 3-6C).

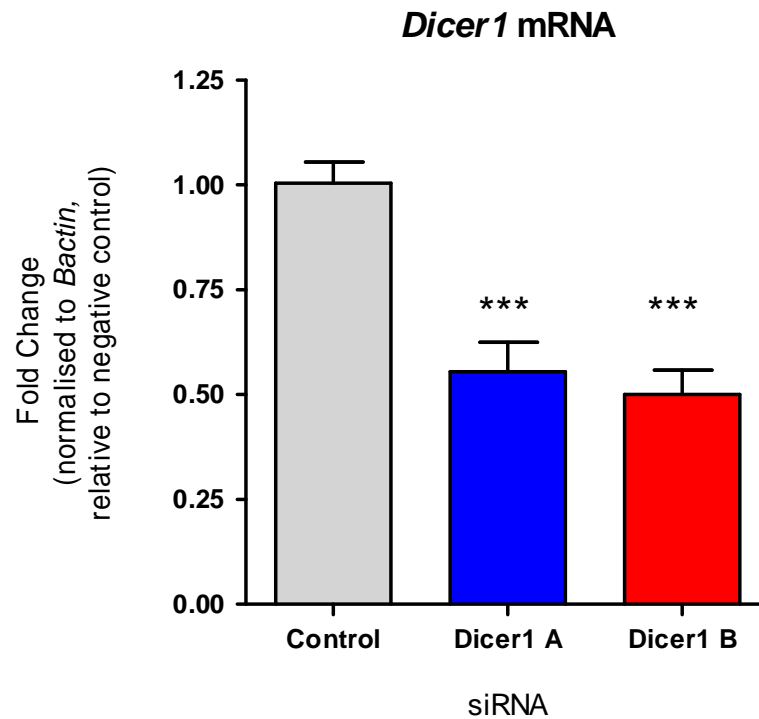


Figure 3-2. Assessment of the Effect of Dicer1 siRNAs on *Dicer1* mRNA in H295R Cells.

H295R cells were transfected with one of two siRNAs targeted against Dicer1, designated Dicer1 A and Dicer1 B or with a scrambled negative control siRNA (final concentration 30 nM). *Dicer1* mRNA were analysed 48 hours post-transfection by qRT-PCR. Cycle threshold values were normalised to *β-actin* mRNA and expressed relative to the control cells. Results represent the mean of three independent biological experiments performed in triplicate; error bars represent SEM. *** $p < 0.001$ compared to control.

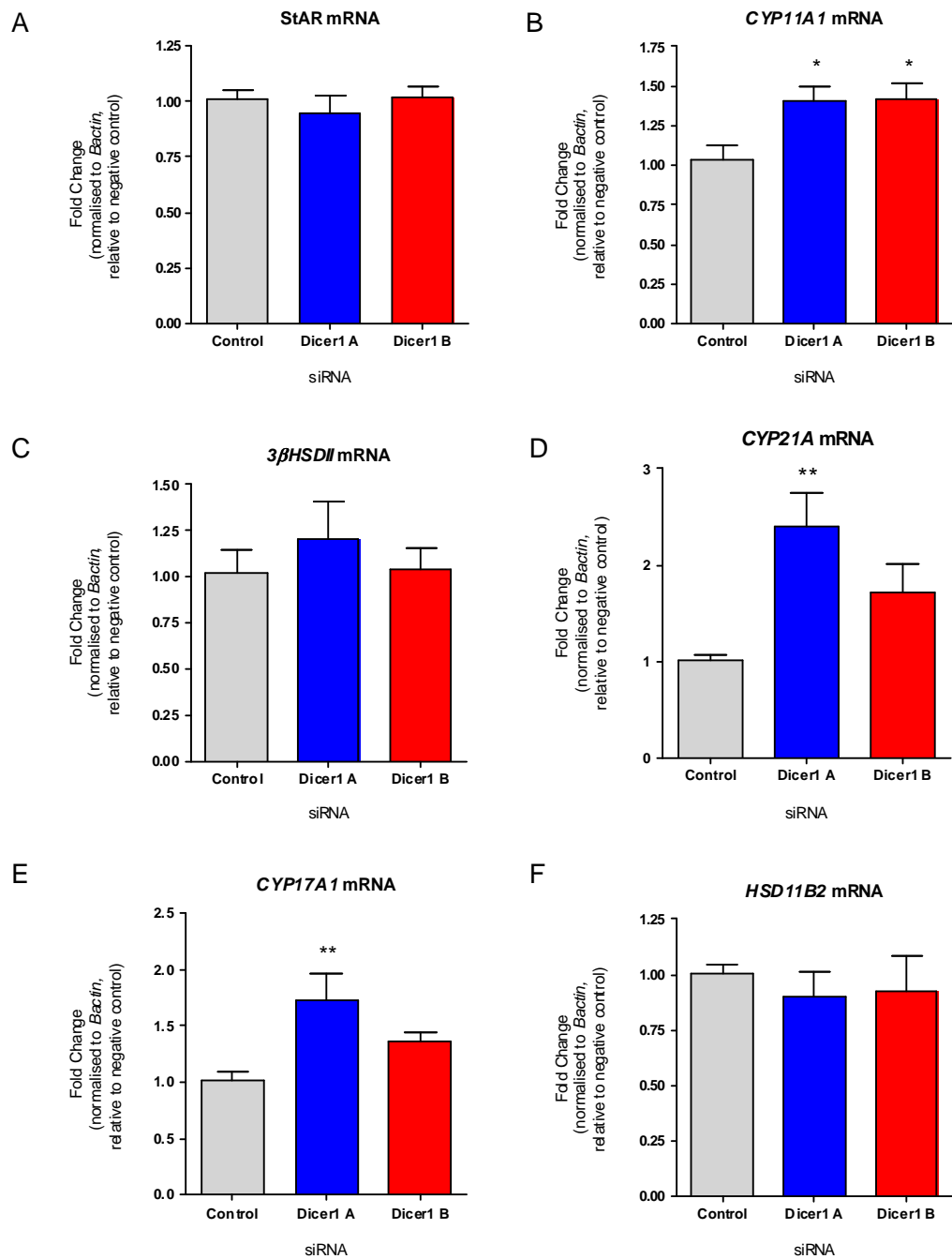


Figure 3-3. Assessment of the Effect of Dicer1 siRNAs on Steroidogenic mRNAs in H295R Cells.

H295R cells were transfected with one of two siRNAs targeted against Dicer1, designated Dicer1 A and Dicer1 B, or with a scrambled negative control siRNA (final concentration 30 nM). *StAR* mRNA (A), *CYP11A1* mRNA (B), *3βHSDII* mRNA (C), *CYP21A1* mRNA (D), *CYP17A1* mRNA (E) and *HSD11B2* mRNA (F) were analysed 48 hours post-transfection by qRT-PCR. Cycle threshold values were normalised to *β-actin* mRNA and expressed relative to the control cells. Results represent the mean of three independent biological experiments performed in triplicate; error bars represent SEM. * p < 0.05, ** p < 0.01 compared to control.

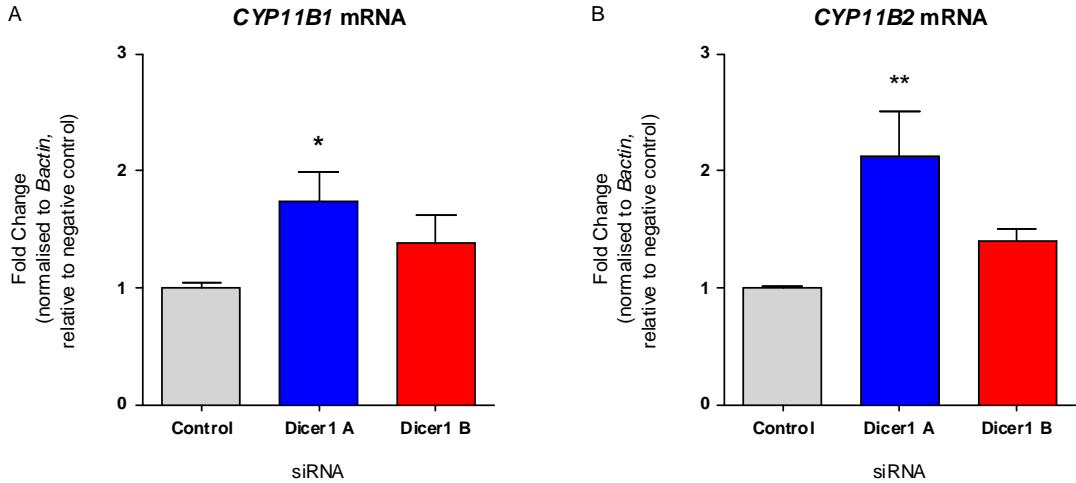


Figure 3-4. Assessment of the Effect of Dicer1 siRNAs on *CYP11B1* and *CYP11B2* mRNA in H295R Cells.

H295R cells were transfected with one of two siRNAs targeted against Dicer1, designated Dicer1 A and Dicer1 B, or with a scrambled negative control siRNA (final concentration 30 nM). *CYP11B1* mRNA (A) and *CYP11B2* mRNA (B) were analysed 48 hours post-transfection by qRT-PCR. Cycle threshold values were normalised to *β -actin* mRNA and expressed relative to the control cells. Results represent the mean of three independent biological experiments performed in triplicate; error bars represent SEM. * $p < 0.05$, ** $p < 0.01$ compared to control.

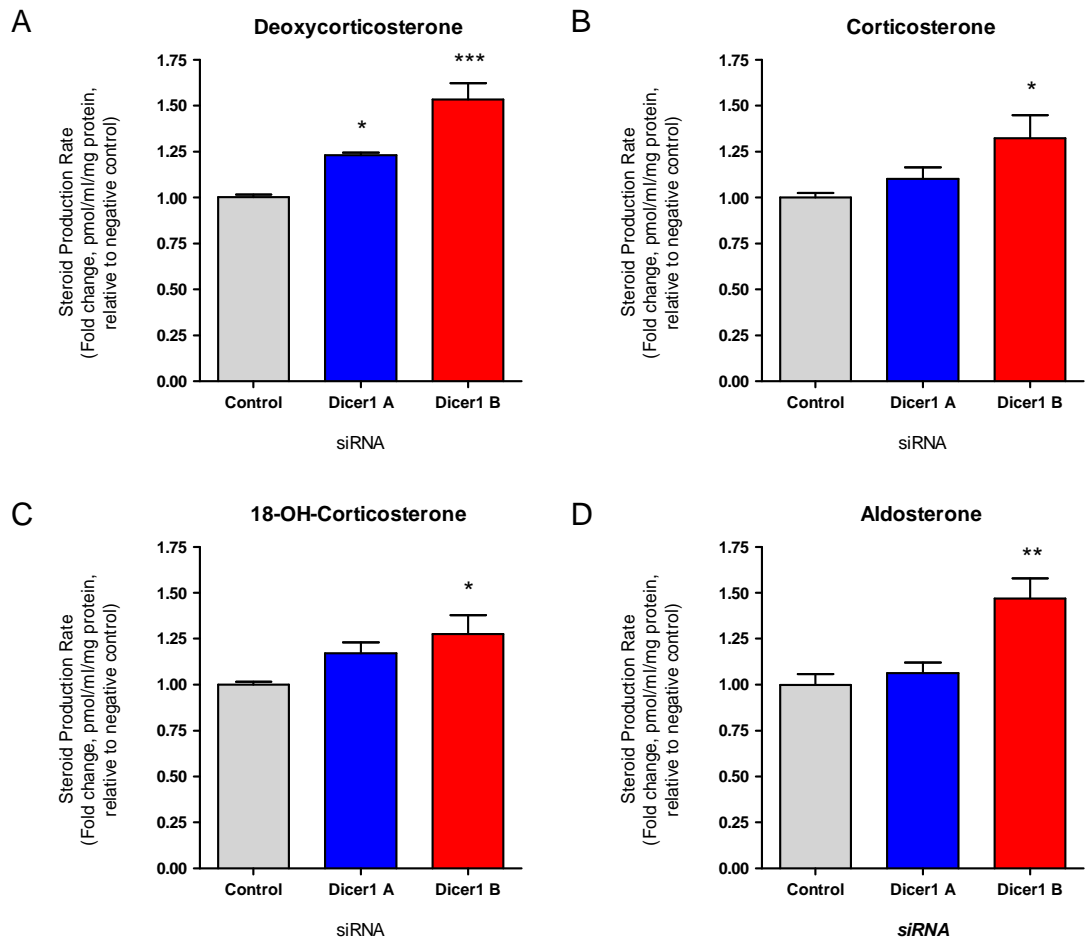


Figure 3-5. Assessment of the Effect of Dicer1 siRNAs on Steroid Production in H295R Cells (aldosterone pathway).

H295R cells were transfected with one of two siRNAs targeted against Dicer1, designated Dicer1 A and Dicer1 B, or with a scrambled negative control siRNA (final concentration 30 nM). 24-Hour steroid secretion was measured in cell growth media by liquid chromatography with tandem mass spectrometry. Steroid production was normalised to total cell protein and expressed as fold-change relative to control cells. Production of deoxycorticosterone (A), corticosterone (B), 18-OH-corticosterone (C) and aldosterone (D) are shown. The results represent the mean of two independent biological experiments performed in replicate groups of 6; error bars represent SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

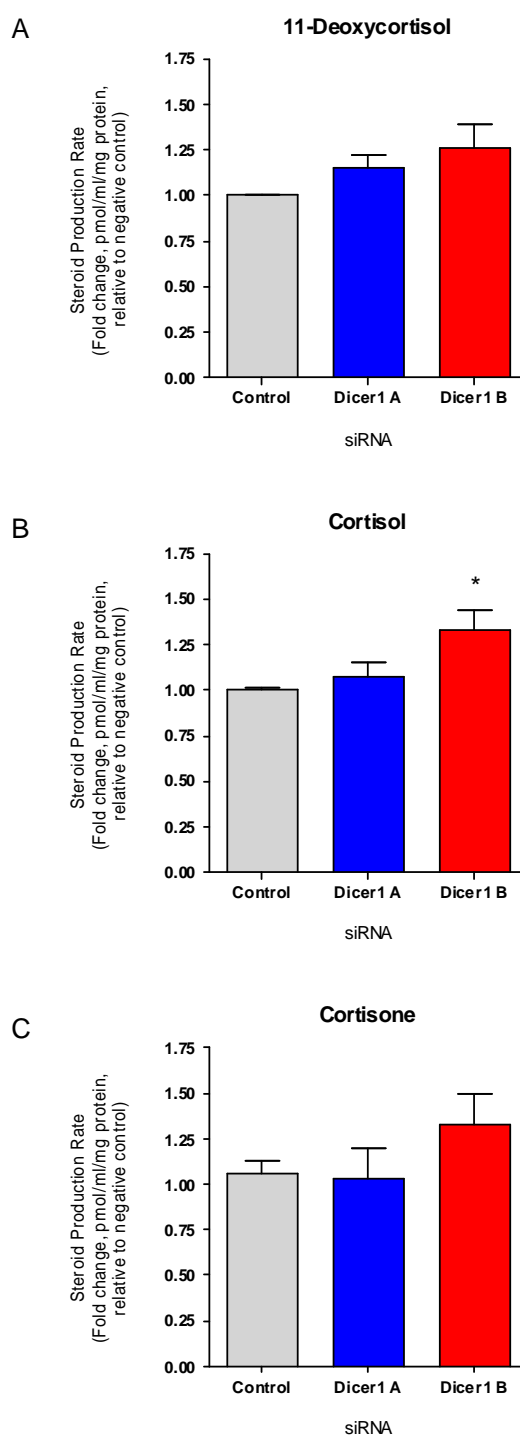


Figure 3-6. Assessment of the Effect of Dicer1 siRNAs on Steroid Production in H295R Cells (cortisol pathway).

H295R cells were transfected with one of two siRNAs targeted against Dicer1, designated Dicer1 A and Dicer1 B, or with a scrambled negative control siRNA (final concentration 30 nM). 24-Hour steroid secretion was measured in cell growth media by liquid chromatography with tandem mass spectrometry. Steroid production was normalised to total cell protein and expressed as fold-change relative to control cells. Production of 11-deoxycortisol (A), cortisol (B) and cortisone (C) are shown. The results represent the mean of two independent biological experiments performed in replicate groups of 6; error bars represent SEM. * $p < 0.05$.

3.4.3 Verification of the pEZX 3'UTR Construct

pEZX-Control plasmid (Figure 2-1) comprises the pEZX backbone contains the firefly and renilla genes and is 7416 base pairs (bp) in size; this is confirmed by restriction endonuclease digestion shown in Figure 3-7. *HindIII*, *SacI* and *SpeI* all linearise the plasmid, yielding a band of that size. *Stu I* enzyme cleaves pEZX-control twice creating two DNA products; 4908 bp and 2508 bp (Figure 3-7).

The pEZX-B1 plasmid comprises the 3'UTR of the *CYP11B1* gene which is approximately 2000 bp in length. Therefore, the pEZX-B1 is 9426 bp in length (Figure 2-1), and this was confirmed by digestion using the *HindIII* enzyme (Figure 3-8A). Both *SacI* and *BamHI* cleave the pEZX-B1 plasmid twice, once in the 3'UTR insert and once in the pEZX backbone. Figure 3-8A shows that *SacI* creates products approximately 7146 bp and 2280 bp in size and *BamHI* creates products 7587 bp and 1839 bp.

The pEZX-B2 plasmid comprises the 3'UTR of the *CYP11B2* gene which is approximately 1500 bp in length. Therefore, the pEZX-B2 is 8835 bp in length (Figure 2-1), and this was confirmed by digestion using the *HindIII* enzyme (Figure 3-8B). *SacI* cleaves the pEZX-B2 plasmid twice, once in the 3'UTR and once in the pEZX backbone and Figure 3-8B shows that *SacI* creates two products approximately 7158 bp and 1677 bp in size. *BamHI* cleaves pEZX-B2 three times, the two larger products 7600 bp and 1839 bp can be visualised in Figure 3-8B, the other DNA fragment at 276 bp was too small to be clearly visible on a 0.7% agarose gel. Finally, *SpeI* digests at either side of the 3'UTR construct creating two products, 7400 bp and 1435 bp in size (Figure 3-8B).

Additionally, the plasmids were directly sequenced (Section 2.7) and examples of the electropherograms generated for each plasmid using SeqScape software are shown in Figure 3-9. Analysis of sequencing spanning the complete insert and adjoining sections of the pEZX backbone verified the identities of the plasmids.

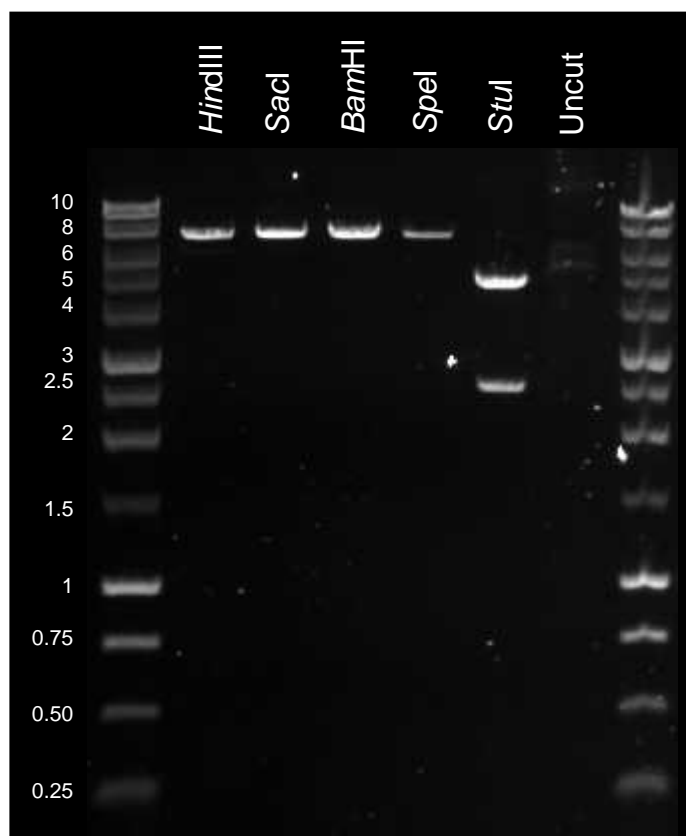


Figure 3-7. Restriction Endonuclease Digestion of pEZX-Control Plasmid.

pEZX-Control plasmid (1 μ g) was digested by various restriction endonuclease and resolved on 0.7% agarose gel. Promega 1 kb ladder was used for size determination; sizes indicated are in kilobase pairs.

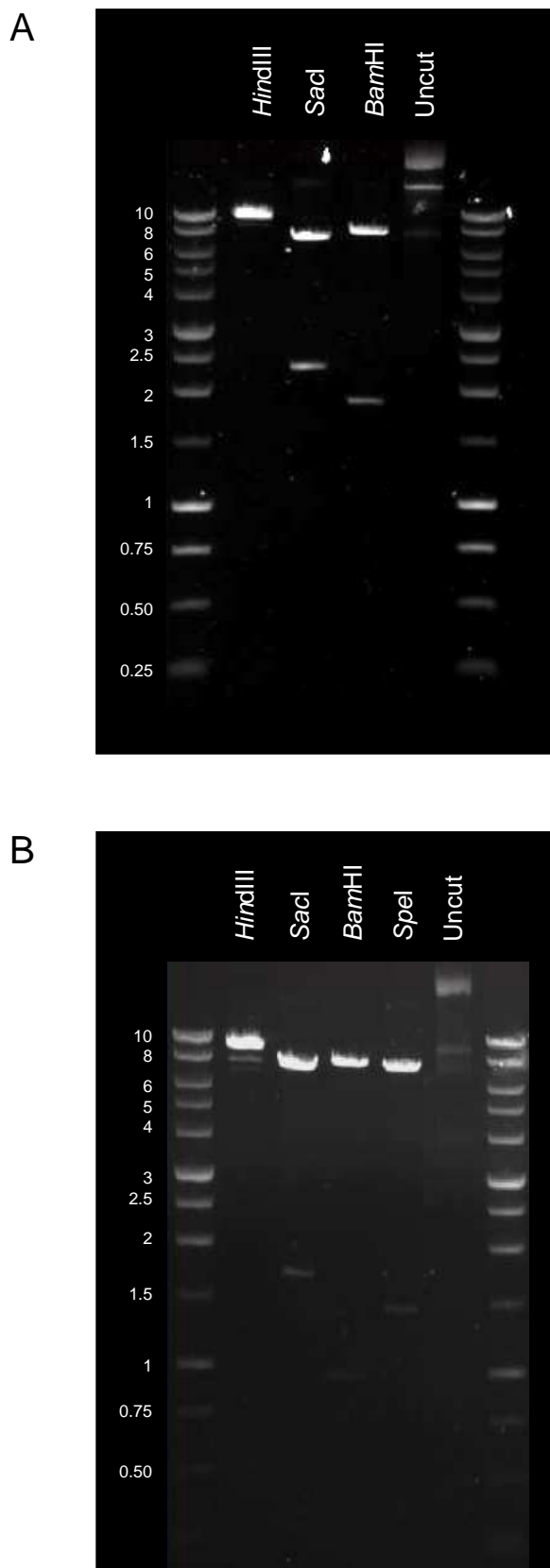


Figure 3-8. Restriction Endonuclease Digestion of pEZX-B1 and pEZX-B2 Plasmid.

pEZX-B1 plasmid (A) or pEZX-B2 plasmid (B) (1 μ g each) was digested by various restriction endonuclease and resolved on 0.7% agarose gel. Promega 1 kb ladder was used for size determination; sizes indicated are in kilobase pairs.

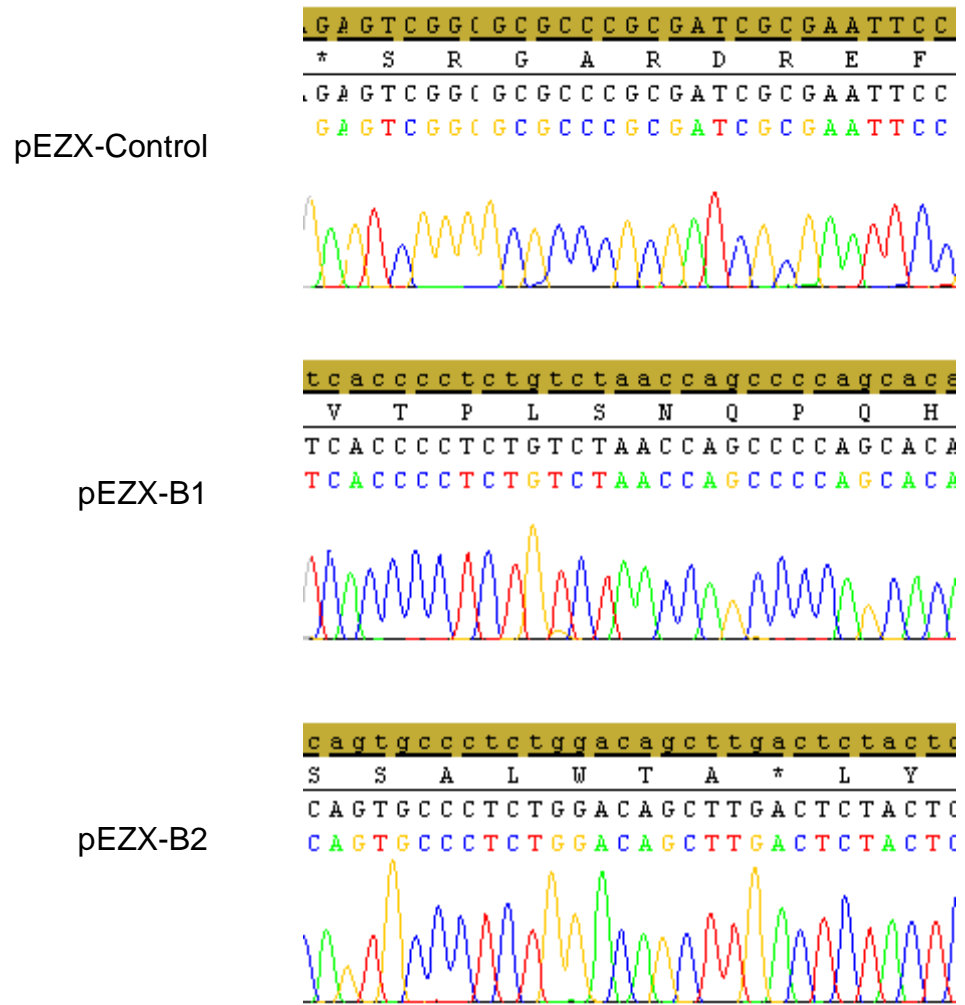


Figure 3-9. Sequence Analysis of pEZX Plasmids.

Examples of electropherogram read from sequence analysis of the pEZX-Control, pEZX-B1 and pEZX-B2 plasmids.

3.4.4 Response of 3'UTR Reporter Construct Activity to AngII Stimulation

pEZX-plasmids (Figure 2-1) were transfected into H295R cells. Firefly and renilla luciferase results (relative light units, RLU) demonstrated that all plasmids were functional and control transfections indicated that the DLRA (Dual Luciferase Reporter Assay) was an appropriate assay for measuring luciferase.

The relative levels of the firefly:renilla ratios of the pEZX-C, pEZX-B1 and pEZX-B2 reporter constructs was assessed after transfecting equal amounts (500ng) of the plasmids into H295R cells (Figure 3-10). The addition of either the 3'UTR of the *CYP11B1* or *CYP11B2* gene to the pEZX backbone caused a significant decrease in the firefly:renilla ratio (0.00048 ± 0.00012 and 0.00050 ± 0.00016) compared to the empty pEZX-C plasmid (0.00220 ± 0.00053). This demonstrates the addition of the 3'UTR of the *CYP11B1* or *CYP11B2* gene is sufficient to cause negative regulation of the firefly mRNA in the H295R cells.

The response the pEZX plasmids was tested in H295R cells stimulated with AngII for 1, 6 or 24 hours (Figure 3-11). There was no significant change in the luciferase ratio for pEZX-control plasmid in the stimulated cells at any time-point (Figure 3-11A). This demonstrates that the pEZX backbone is not responsive to the corticosteroid trophin, AngII. There were no significant differences in the luciferase ratio of the pEZX-B1 plasmid after stimulation with AngII for 1 or 6 hours ($105.93 \pm 5.62\%$ and $101.39 \pm 2.88\%$, respectively; Figure 3-11B). When stimulated with AngII for 24 hr, the luciferase ratio increased to $192.70 \pm 28.04\%$ of non-stimulated cells ($p < 0.05$; Figure 3-11B). The luciferase ratio of pEZX-B2 plasmid did not significantly differ in AngII-stimulated H295R cells at 1 or 6 hours ($101.82 \pm 2.41\%$ and $100.67 \pm 3.85\%$, respectively; Figure 3-11C). 24-Hour AngII stimulation increased the luciferase ratio to $220.58 \pm 43.52\%$ ($p < 0.05$; Figure 3-11C).

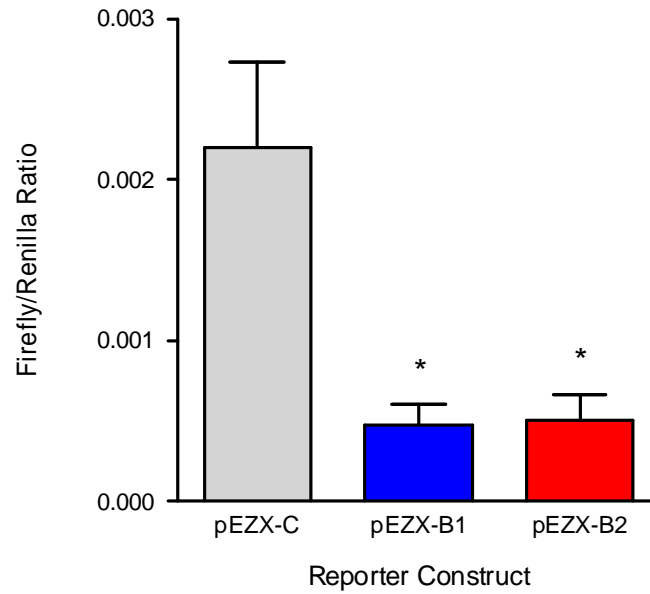


Figure 3-10. Assessment of Relative Levels of Reporter Construct Activities.

H295R cells were transfected with the pEZX control, pEZX-B1 or pEZX-B2 then lysed after 48 hours. Firefly and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay Kit and normalised for transfection efficiency using the ratio of firefly to renilla luminescence. Results represent the mean of at least three independent biological experiments performed in quadruplicate; error bars represent SEM. Statistical analysis was a 1-way ANOVA with Bonferroni's Multiple Comparison correction. * $p < 0.05$ relative to pEZX-C.

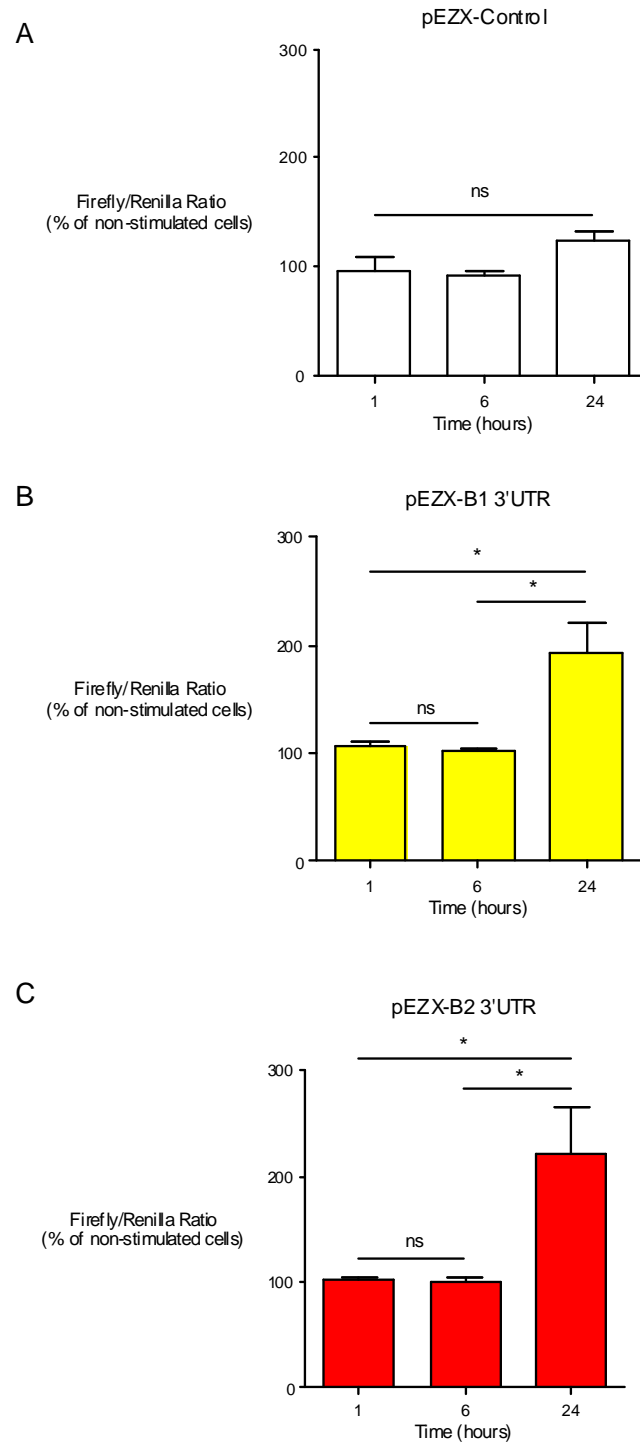


Figure 3-11. Response of pEZX 3'UTR Reporter Constructs to Angiotensin II Stimulation

H295R cells were transfected with the pEZX control (A), pEZX-B1 (B) or pEZX-B2(C) then lysed after 48 hours. Prior to lysis, medium was replaced with AngII (10 nM) supplemented medium for 1, 6 or 24 hrs. Firefly and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay Kit and normalised for transfection efficiency using the ratio of firefly to renilla luminescence. The results for the stimulated cells were expressed as a percentage of time matched non-stimulated control cells. Results represent the mean of at least three independent biological experiments performed in quadruplicate; error bars represent SEM. Statistical analysis was a 1-way ANOVA with Bonferroni's Multiple Comparison correction. ns; non-significant, * $p < 0.05$.

3.5 Discussion

The experiments presented in this chapter investigated if miRNAs mediate regulation of corticosteroid-related mRNA levels and steroid production in the adrenocortical cell line, H295R. To achieve this, miRNA levels were reduced using *Dicer1*-targeted siRNA and the activation of the *CYP11B1* and *CYP11B2* 3'UTR investigated in response to the classical corticotrophic peptide, AngII.

The first stage involved transient siRNA transfection into H295R cells; the transfection protocol and the positive control siRNA molecule were shown to be successful in altering *GAPDH* mRNA levels. Therefore, a method was successfully developed for small RNA transfection into the H295R cells, and was adopted for the subsequent siRNA transfections. The siRNA molecules were purchased as pre-validated siRNAs from Applied Biosciences and this study confirmed *Dicer1* mRNA silencing in H295R cells. The magnitude of *Dicer1* mRNA reduction was relatively small: 0.5 fold change relative to control cells. This is equivalent to reducing them to approximately 75% of their original levels. siRNA-silencing only affects nascent mRNAs therefore, *Dicer1* enzyme synthesised prior to transfection will still be present and functional within cells, assessing the level of Dicer protein by western blot analysis would help quantify the impact of this. The reduction in *Dicer1* mRNA levels is slightly less than that reported in the HeLa cell line by the manufacturer; this is likely to be caused by differences in the cell types (H295R cells are notoriously difficult to transfect) and altered concentrations of siRNA.

Despite the modest decrease in *Dicer1* mRNA, a reduction in miRNA levels would still be expected; a change of similar magnitude has already been shown to reduce levels of several miRNAs (Lee *et al.*, 2006). Yet, this protocol does not eliminate all miRNAs as some Dicer activity remains and the turnover rate of most miRNAs is still unknown (Section 1.5.2.4). To determine miRNA levels in *Dicer1* knockdown cells, it would be necessary to perform miRNA microarray, qRT-PCR or northern blot analyses. Moreover, recent reports suggest that miRNAs may be formed by a *Dicer1*-independent pathway, and this has been demonstrated for miR-451 *in vitro* (Yang *et al.*, 2010) and in mice models (Cheloufi *et al.*, 2010; Cifuentes *et al.*, 2010). In this relatively newly-discovered pathway pre-miR-451 is cleaved by Argonaut2, the importance of this alternative

pathway remains to be evaluated. In summary, transient transfection of Dicer1-targeted siRNA in H295R cells reduces maturation of novel miRNAs and thus serves as a good model to test the potential impact of miRNA-regulation on corticosteroidogenesis.

The mRNA levels of several key enzymes in the adrenal steroid production pathway were assessed cells transfected with in Dicer1-targeted siRNA. Reduction of *Dicer1* mRNA did not change the relative levels of *StAR*, *3BHSDII* or *HSD11B2* mRNA, but did increase the levels of the CYP450 mRNA analysed (Sections 1.2.3 and 1.2.4). This indicates that reducing miRNAs leads to the de-repression of CYP450 expression in H295R cells. The change in mRNA level was approximately 1.5 - 2 fold depending on the gene and this is typical of the fine-tuning effect usually exerted by miRNAs (Sevignani *et al.*, 2006). Significant increases in the mRNA level of CYP450 mRNAs, other than *CYP11A1*, were only achieved with the Dicer1 A siRNA. Although an increase in these mRNA was always observed with the Dicer1 B siRNA, the magnitude of change was smaller or the error slightly larger relative to the Dicer1 A siRNA, meaning a statistical significant change was not observed. One possible explanation for this difference is that each siRNA targets different regions of *Dicer1* mRNA (Appendix; Figure 8-7-1), and this may alter the mechanism and/or efficiency of silencing. This study validated two separate Dicer-targeting siRNAs for this purpose but could have also co-transfected two Dicer-targeting siRNAs.

The majority of steroids produced from H295R cells are androgens although they also retain corticosteroid-synthesising abilities. Dicer1-targeted siRNA transfected cells had increased production of DOC, B, 18-OH-B and aldosterone. Together, these steroid results and those measuring mRNA indicate that expression of the CYP21A1 and CYP11B2 enzymes may be regulated by miRNAs. Moreover, cortisol production was increased, suggesting miRNA regulation of *CYP11B1* transcript abundance. The cortisol precursor, S, was not significantly increased in the knockdown cells, which is inconsistent with the role of *CYP21A1* mRNA results and increased DOC production. It was not possible to assess the enzyme efficiency by comparing the relative levels of precursor and substrate (for example, the S:F ratio) as the sample size and the relative amounts of steroid produced were too small. The level of *HSD11B2* mRNA was unaffected by Dicer1-targeted siRNA, which might be expected given that the conversion of

cortisol to cortisone is of greater relevance to peripheral tissues, rather than in the adrenal gland.

The focus of this thesis is to investigate the role of miRNAs in adrenal corticosteroidogenesis. Therefore, the majority of work described in subsequent chapters will be concerned with the *CYP11B1* and *CYP11B2* genes. The above experiments undoubtedly support a role for miRNA regulation in these genes' expression and the generation of their relevant steroid products. To investigate whether this is mediated by direct action on the 3'UTR of *CYP11B1* and/or *CYP11B2* mRNAs, reporter constructs containing the 3'UTR of these genes were used. Initial experiments demonstrated that there was significant repression of the luciferase mRNA compared to a no-3'UTR control construct (as indicated by the relative firefly:renilla activity ratios), this supports a role for miRNA-mediated regulation acting at the 3'UTR of both the *CYP11B1* and *CYP11B2* genes.

The construct were then further tested in H295R cells to determine whether miRNA repression could be overcome by stimulation of H295R cells with AngII, which has repeatedly been shown to increase *CYP11B2* and, to a lesser extent, *CYP11B1* expression (Denner *et al.*, 1996). The presence of *CYP11B1* or *CYP11B2* 3'UTR was sufficient to increase reporter construct activity levels in response to AngII stimulation. This increase was only observed in cells stimulated for 24 hours and no response was observed in the control plasmid, which lacked a 3'UTR. Thus, the observed increase in activity can be attributed to the *CYP11B1* or *CYP11B2* 3'UTRs. This is consistent with AngII stimulating *CYP11B2* or *CYP11B1* protein synthesis and de-repression of the 3'UTR by miRNAs. Although the influence of AngII on the expression levels of miRNAs cannot be determined from these experiments, a previous study using H295R cells found that miR-21 was increased following AngII stimulation for 6, 12 and 24 hours (Romero *et al.*, 2008) suggesting that the regulation of certain some adrenal miRNAs is sensitive to AngII. However, the expression profile of adrenal miRNAs and their response to AngII has not been fully determined and it is, therefore, difficult to ascertain what regulatory mechanism dominates. Therefore, further experiments are required in order to determine whether the observed changes in reporter gene activity are attributable to modulation of miRNA levels or to indirect effects such as alterations in Dicer2 levels.

In conclusion, these studies have provided evidence of a role for miRNA-mediated regulation of CYP450 expression, which has an impact on adrenal corticosteroidogenesis and therefore, appears to be of biological significance. The identity or contribution of individual miRNAs cannot be assessed on the basis of these experiments, but they clearly support further investigation.

4 Normal Adrenal Gland microRNA Profiling and Target-Site Prediction

4.1 Introduction

To investigate miRNA-mediated regulation of a particular mRNA, the investigator is required to have an understanding of the expression of miRNAs in the tissue or cell type that expresses the mRNA. It is then necessary to understand whether miRNA targeting of the mRNA is feasible by identifying miRNA-binding sites in its 3'UTR. The miRNA expression profile of a tissue or a cell can only be properly determined by experimental analysis, whether by qRT-PCR, miRNA microarray analysis, northern blot or deep-sequencing. However, computational strategies are available that either map experimentally validated genomic locations of miRNAs or facilitate prediction of the genetic location of novel miRNA species. Using this strategy offers the investigator the opportunity to perform a relatively quick and cost-effective analysis prior to investing in expensive 'wet-lab' techniques that may take time and require precious tissue samples.

Computational methods designed to identify genomic regions that encode pri-miR transcripts have been developed based on common features of miRNAs, for example: structural properties such as hairpin length, mismatched base-pair bulge size and thermodynamic stability or pri-miR sequence properties (Bentwich *et al.*, 2005; Bentwich, 2005). Frequently the algorithms used rely on evolutionary sequence conservation for identification of miRNAs; this is valid as many miRNAs are well conserved across species (Berezikov *et al.*, 2005). However, this method is limited by the accuracy of the algorithm and also by miRNAs which are not conserved, therefore it fails to detect species-specific miRNAs (Bentwich *et al.*, 2005). Validation of miRNA prediction is required and this can be achieved by various methods of direct sequencing. More recently, deep-sequencing has proved successful for identifying novel miRNAs. This involves sequencing large fractions of RNA of unknown function, with the aim of identifying novel regions which resemble a pri-miRNA or pre-miRNA (Friedlander *et al.*, 2008).

In circumstances where a specific gene target is unknown (*e.g.* in studies of defined disease phenotypes or of cell differentiation), a gene expression microarray is often performed concurrently with miRNA expression analysis. The

aim is to identify negatively correlated miRNA:mRNA combinations. This strategy is complicated as a single miRNA can regulate many genes.

In addition, *in silico* methods have been developed to predict mRNA targets for miRNAs. However, as will be discussed below, this is highly complex and has proven to be problematic. There are numerous miRNA databases and target-prediction algorithms but in general, the validation rate is low, possibly because miRNAs are promiscuous and believed to bind to hundreds of different targets (Lewis *et al.*, 2003). Further, miRNA regulation does not require perfect base-pairing complementarity of miRNA to mRNA and, therefore, target identification by simple BLAST (Basic Local Alignment Search Tool) base-pairing analysis cannot be used (Rehmsmeier *et al.*, 2004). Moreover, this leads to the formation of complex RNA secondary structure. Experimental testing and validation of miRNA target sites has provided a learning dataset against which algorithms can be compared; this has improved the accuracy of the algorithms and of the target site predictions. It has also identified a region known as the ‘seed site’, which refers to nucleotides 2-7 of the miRNA from the 5’ end (Figure 4-1). Watson-Crick base-pairing in this region is often evolutionarily conserved and leads to a higher rate of positive target predictions being generated (Lewis *et al.*, 2005). Perfect complementarity in this region is referred to as a 6mer site (Figure 4-1). Further analysis has identified that a base-pairing at nucleotide 8 further increases target recognition, as does an adenosine nucleotide at position 1 of the miRNA; instances of these are termed 7mer-m8 site and 7mer-A1 sites, respectively (Figure 4-1). miRNA:mRNA pairings which have both of these additional features are termed 8mer sites (Figure 4-1) (Lewis *et al.*, 2003; Lewis *et al.*, 2005). Some miRNAs have common sequences at the seed site, having evolved from a common ancestor, and are collectively known as a miRNA family (Griffiths-Jones *et al.*, 2003). Seed site base-pairing forms one of the strongest criteria for several target prediction databases, increasing the reliability and reducing the rate of false positive predictions (Bartel, 2009). Moreover, base-pairing in the seed region is sufficient to elicit miRNA-regulation as even a single nucleotide polymorphism in the seed region can disrupt miRNA function (Doench and Sharp, 2004).

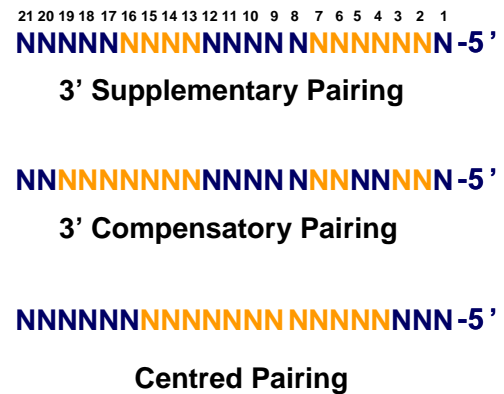
Seed SitesAlternative Base-Pairing

Figure 4-1. miRNA Target Recognition Base Pairing Requirements.

miRNA seed site base-pairing is shown in the left panel. Nucleotides (N) are numbered from the 5' end of the miRNA. Yellow nucleotides (N) represent Watson-Crick matches to the 3'UTR of mRNA. Red bases are the additional matches that increase target recognition and miRNA silencing and include, adenosine (A) at position 1. The right hand panel shows nucleotide matches in the 3' end that can be present with or without a canonical seed site. The bottom miRNA illustrates centered pairing.

In addition to the seed region, pairing of the mRNA target to other miRNA bases can affect its target recognition and silencing. Complementary binding at the 3' end of the miRNA (Figure 4-1) can enhance miRNA function for targets which also have a strong seed site complementarity; this is known as supplementary pairing, and refers to continuous base-pairing at nucleotides 13-16 (Grimson *et al.*, 2007). Further, enhanced base-pairing, typically from nucleotides 13-19, can make up for mismatched bases within the seed site and is known as compensatory pairing (Figure 4-1) (Grimson *et al.*, 2007; Bartel, 2009). Recently, miRNA-mediated regulation lacking both a traditional seed site and 3' complementarity has been identified. This involves alternative base-pairing of 11 or 12 continuous bases in the centre region of the miRNA (nucleotides 4-15) to a target mRNA (Figure 4-1). At high Mg^{2+} concentrations this is sufficient to cause miRNA-mediated silencing (Shin *et al.*, 2010).

Another parameter used by prediction algorithms is the degree of evolutionary sequence conservation of the miRNA species and of the target sites. A higher degree of sequence conservation is associated with a decreased number of false-positive miRNA target predictions (Lewis *et al.*, 2005; Bartel, 2009). Moreover, the conservation of most miRNA target sites identified to date is higher than would be expected by chance. Thus, they are believed to be under selective pressure and indicates a strong likelihood of biological function. However, the stringency of conservation used by bioinformatic analyses may miss miRNA-targets that are species-specific, and it has been estimated that 30% of validated miRNA:mRNA interactions are not conserved (Sethupathy *et al.*, 2006).

Finally, most target prediction algorithms do not account for RNA secondary structure of mRNA or for miRNAs which will affect the ability for base-pairing to occur efficiently; this may contribute to a poor rate of target validation. Prediction of secondary structure and the minimum free-energy may be useful for identifying interactions that are physiologically likely to occur (Robins *et al.*, 2005; Kertesz *et al.*, 2007). However, modelling RNA secondary structure is not 100% accurate and removing this feature from one of the databases does not change the specificity of target prediction (Lewis *et al.*, 2005; Bartel, 2009).

Despite the problems outlined above, target site prediction databases still provide the investigator with useful information regarding miRNA-targeting

4.2 Aims

The aims of this study were to investigate the genomic location of pri-miRNA sequences; to utilise bioinformatic prediction algorithms to identify putative miRNA binding sites in the 3'UTR of the *CYP11B1* and *CYP11B2* genes; and to establish the expression profile of miRNAs in non-tumorous (normal) adrenal glands and then to cross-reference the target and profiling data to compile a list of candidate miRNAs that may be important in the regulation of the *CYP11B1* and *CYP11B2* genes.

4.3 Materials and Methods

4.3.1 Bioinformatic Analysis

4.3.1.1 Identification of the Genomic Location of miRNAs

The genomic co-ordinates, strand location and mRNA transcript length of the human genes encoding corticosteroidogenic enzymes were identified using the Ensembl Genome Browser (release 61 - Feb. 2011). These details were used to cross-reference with known miRNA precursor sequences that are, mapped and stored in the miRBase database (release 16 - Sept. 2010).

4.3.1.2 Investigation of the Evolutionary Sequence Conservation of the *CYP11B1* and *CYP11B2* 3'UTR

Analysis of the evolutionary sequence conservation of the 3'UTR of the *CYP11B1* and *CYP11B2* mRNA was performed using the UCSC Genome Browser Gateway (Feb. 2009 assembly). For comparison, 3 non-human primates (chimpanzee, gorilla and rhesus macaque) and 5 other mammals (mouse, rat, rabbit, cow and dog) were used, and a graphical representation was generated.

4.3.1.3 Structural Prediction of the 3'UTR of *CYP11B1* and *CYP11B2*

The secondary structure of the 3'UTR of *CYP11B1* and *CYP11B2* genes was predicted using the RNAFold program from the Vienna RNA Package (version 1.8.5). This was achieved by entering gene information from the UCSC Genome Browser Gateway system and exporting pictorial results and free-energy predictions from the software program.

4.3.1.4 Bioinformatic miRNA Target Site Prediction

A combinational approach using five databases was applied to identify putative miRNA binding sites in mRNAs involved with corticosteroidogenesis. The databases are listed in Table 4-1. Where required, the full-length human transcript was selected and conservation was set to low. All other parameters were left at default settings. Database predictions for each gene from the

database were recorded, pooled and duplicate results removed to create a list of putative miRNA binding sites for each gene.

4.3.2 miRNA Expression Profiling of Normal Adrenal Glands

4.3.2.1 miRNA Microarray Analysis of miRNA Expression

Total RNA was isolated from four frozen non-tumorous adrenal glands (section 2.2.1) and quantified as described in sections 2.2.4 and 2.2.5. Samples were shipped to the microarray provider (LC Sciences, Houston, Texas) and the array performed (Section 2.3). A background threshold cut-off of 500 arbitrary units was used as recommended by the array chip manufacturer; miRNAs expressed at levels greater than this threshold were deemed to be expressed in the adrenal gland.

4.3.2.2 qRT-PCR Validation of miRNA Expression

Twenty miRNAs with expression levels greater than the threshold were validated by qRT-PCR. This was performed by LC Sciences (Houston, Texas, U.S.A.) using the Applied Biosciences miRNA Taqman[®] Assay and the method described in Section 2.4.2 and 2.5.2. Two small nucleolar housekeeping RNAs, RNU48 (SNORD48) and U47 (SNORD47), were also run in the assay. RNU48 was the most stably expressed and, therefore, was used for normalisation purposes.

4.3.3 Statistical Analysis

All data are presented as mean \pm SEM. the correlation of the microarray expression data and the qRT-PCR validation was tested using Prism 4.0 Graph Pad software.

Table 4-1. Bioinformatic Databases Used for Screening miRNA Target Sites.

| Database | Version | Algorithm | Reference |
|---|-----------------|---|---|
| microrna.org | August 2010 | miRanda - miRSVR (Support Vector Regression) | (John <i>et al.</i> , 2004;Betel <i>et al.</i> , 2010) |
| miR-viewer | June 2005 | miRanda | http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl |
| TargetScan | 5.1, April 2009 | | (Lewis <i>et al.</i> , 2005;Grimson <i>et al.</i> , 2007;Friedman <i>et al.</i> , 2009) |
| MicroCosm Targets (formally miRBase) | 5 | | (Griffiths-Jones <i>et al.</i> , 2006;Griffiths-Jones <i>et al.</i> , 2008) |
| TarBase | 5 | | (Papadopoulos <i>et al.</i> , 2009) |

4.4 Results

4.4.1 Genomic Prediction of pre-miRNA Coding Regions

No miRNA precursor sequences are currently mapped to the intronic regions of the nine selected corticosteroidogenic genes analysed (Table 4-2). The most proximal miRNA sequence to any of the genes was hsa-miR-146b, which is coded on the forward strand of human chromosome 10. It is approximately 300,000 bases downstream of the *CYP17A1* gene at co-ordinates 104,196,269 to 104,196,341. This miRNA is located in an intergenic region and is surrounded by several other protein-coding genes.

Table 4-2. Genetic Location of Human Corticosteroid Genes and the Predicted miRNAs Sequences.

| Gene | Chromosome Location | Start Co-ordinates | End Co-ordinates | Size (Bases) | Number of miRNA Precursor Sequences |
|----------------|---------------------|--------------------|------------------|--------------|-------------------------------------|
| <i>CYP11B1</i> | 8 | 143,953,772 | 143,961,262 | 7,490 | 0 |
| <i>CYP11B2</i> | 8 | 143,991,975 | 143,999,259 | 7,284 | 0 |
| <i>CYP11A1</i> | 15 | 74,630,100 | 74,660,081 | 29,981 | 0 |
| <i>CYP17A1</i> | 10 | 104,590,288 | 104,597,290 | 7,002 | 0 |
| <i>CYP21A2</i> | 6 | 31,965,200 | 32,001,297 | 3,405 | 0 |
| <i>HSD3B2</i> | 1 | 119,957,673 | 119,965,657 | 7,984 | 0 |
| <i>STAR</i> | 8 | 38,000,226 | 38,008,783 | 8,557 | 0 |
| <i>HSD11B1</i> | 1 | 209,859,510 | 209,908,295 | 48,785 | 0 |
| <i>HSD11B2</i> | 16 | 67,465,041 | 67,471,456 | 6,415 | 0 |

Start and End co-ordinated refer to transcription start and stop sites.

4.4.2 Analysis of the 3'UTR of the *CYP11B1* and *CYP11B2* Genes

The predicted secondary structure of the *CYP11B1* and *CYP11B2* 3'UTRs is shown in Figure 4-2. Secondary structure can affect the accessibility of a miRNA to its target-site; if the energy required for binding is too high then binding would be unlikely. This analysis does not evaluate any miRNA binding sites but does give an estimation of the unbound secondary structure of the native 3'UTR, which appears not to have too many complex folds (Figure 4-2).

The evolutionary sequence conservation of the 3'UTR of the *CYP11B1* and *CYP11B2* 3'UTRs was assessed using the comparative genomic analysis available within the UCSC Genome browser. The regions (highlighted in blue and indicated by red dashed lines) were compared to other mammals and the results can be seen in Figure 4-3. The 3'UTR of both genes is well conserved across the higher mammals, including the chimp, gorilla and rhesus macaque, as indicated by the solid black bars, which indicate sequence similarity. The degree of similarity is substantially reduced for the mouse, rat, rabbit, cow and dog sequences (Figure 4-3).

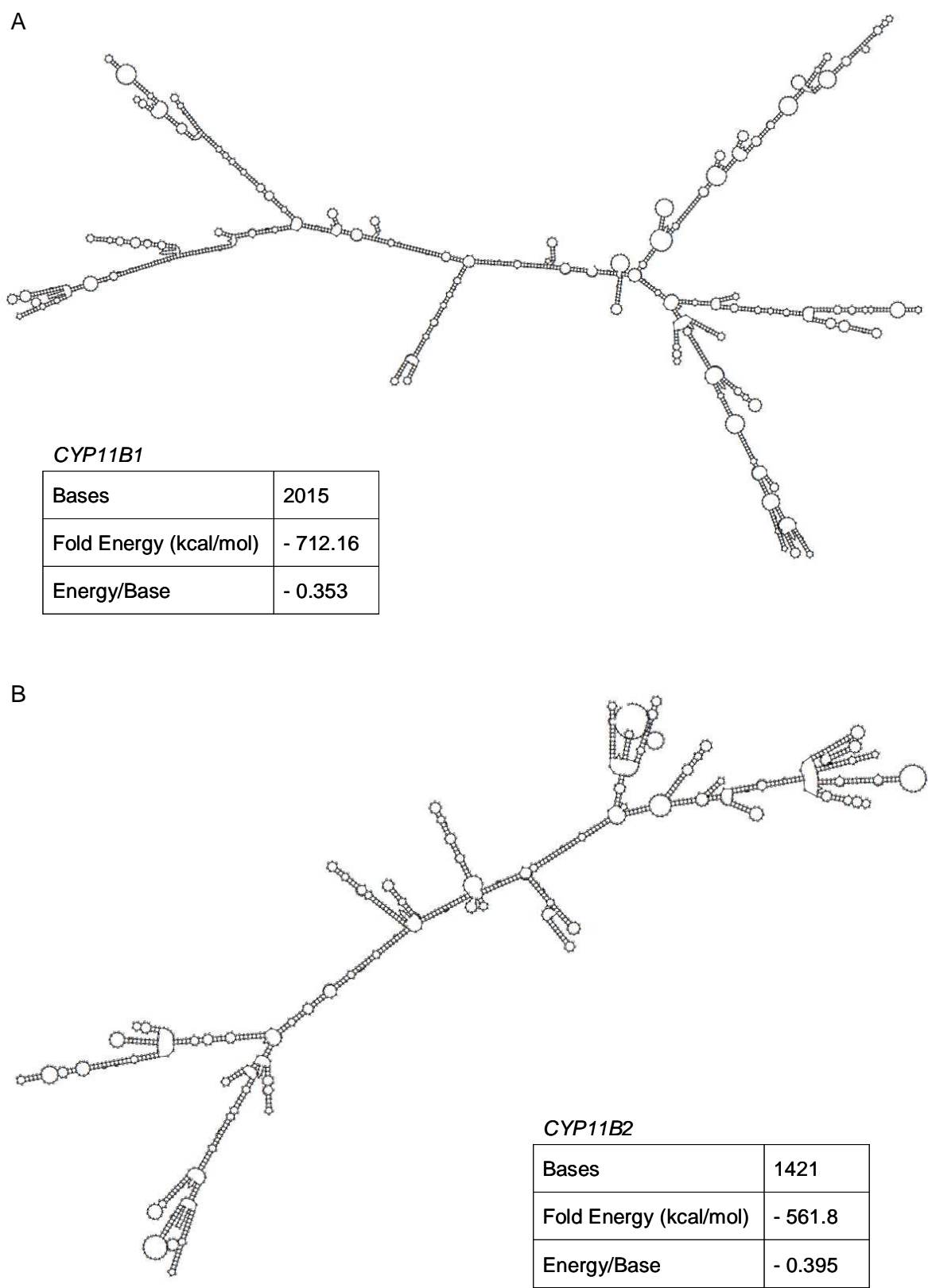


Figure 4-2. Predicted RNA Secondary Structure of the 3'UTR of the *CYP11B1* and *CYP11B2* Genes.

Predicted structure of the 3'UTR of *CYP11B1* (A) and *CYP11B2* (B) from the UCSC Genome Browser Gateway and the Vienna RNA Package.

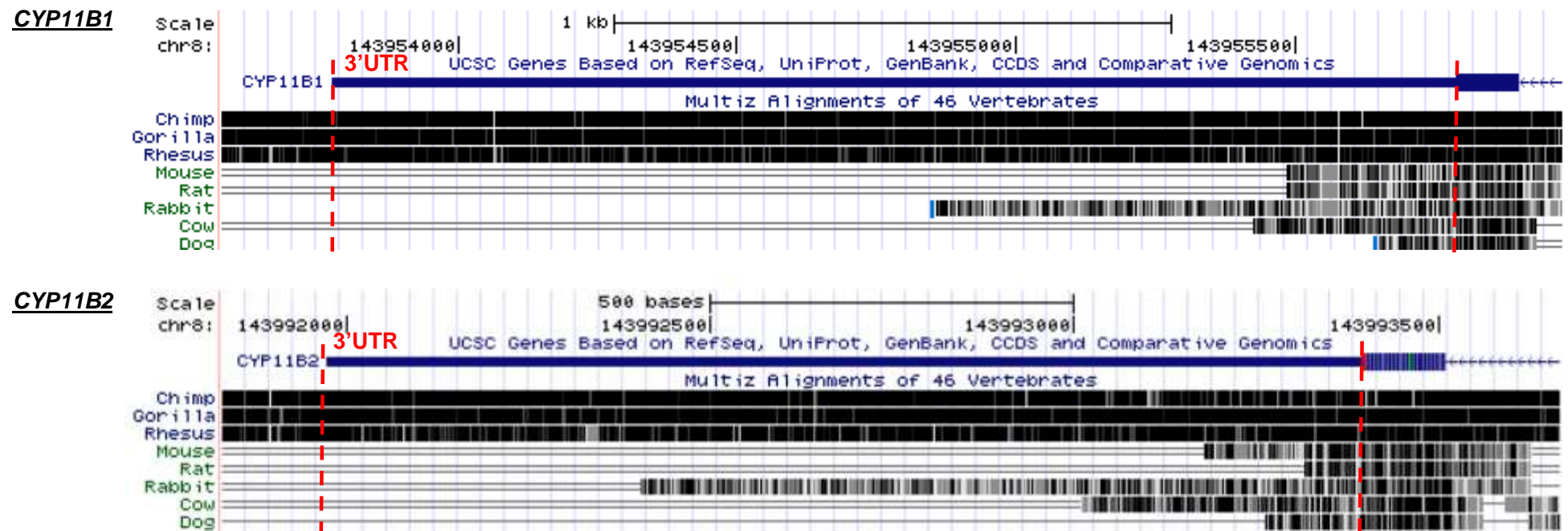


Figure 4-3. Mammalian Evolutionary Conservation of the 3'UTR of the *CYP11B1* and *CYP11B2* Genes.

The sequence conservation, generated by the UCSC Genome Browser for the *CYP11B1* (top) and *CYP11B2* (bottom) 3'UTR, shown by the blue bar and indicated by the dashed red lines. Conservation is represented by black bars for the species listed on the left-hand side and is defined as per default settings of the Genome Browser.

4.4.3 Identification of Putative miRNA Binding Sites

Table 4-3 lists the 3'UTR length for each of the selected corticosteroidogenic genes. Of the genes chosen, the *CYP11B1* and *CYP11B2* genes have the longest 3'UTR at 2022 base-pairs and 1428 base-pairs, respectively. This region comprises a large proportion of the total size of each mRNA transcript (Table 4-2) and the 3'UTR is relatively large in comparison to those of the other genes especially *CYP11A1* gene, whose 3'UTR comprises less than 1% of its total size (Table 4-3).

The five databases listed in Table 4-1 were searched, in turn, to identify putative miRNA binding sites in each of the selected corticosteroidogenic genes. The Tarbase database relies on validated targets and produced no miRNA target predictions for any of the genes investigated. Table 4-3 summarises the results generated from the other four databases. It lists the number of target sites predicted by each algorithm, with the number of individual miRNAs in parentheses. The collated results are shown in the final column; this excludes any duplicate predictions made by one or more algorithm.

The longest (*CYP11B1*) and shortest (*CYP17A1*) 3'UTRs match the highest and lowest number of miRNA prediction, respectively. However, the numbers of predictions do not correlate well with the size of the 3'UTR for other mRNAs. The *CYP11B2* 3'UTR has approximately the same number of predicted binding sites as *StAR* and *HSD11B1* despite being over twice as long as that of the other genes.

The extent of sequence similarity between the *CYP11B1* and *CYP11B2* genes prompted the comparison of predicted miRNA target sites. There was a large degree of similarity; in total 84 putative miRNA binding sites common to both genes are listed in Table 4-5. The identities of the 119 miRNAs predicted to bind to *CYP11B1* only are listed in Table 4-4 and the 88 miRNAs with putative binding sites to *CYP11B2* are listed in Table 4-6.

Table 4-3. Bioinformatic miRNA Target Site Predictions for Corticosteroidogenic Genes

| Gene | 3'UTR Length (Base pairs) | Microrna.org | miR- viewer | TargetScan | MicroCosm Targets | Unique miRNAs with Binding Sites |
|---------------------------------|------------------------------|--------------|-------------|------------|----------------------|-------------------------------------|
| <i>CYP11B1</i> | 2022 | 150 (142) | 4 (4) | 196 (156) | 33 (30) | 202 |
| <i>CYP11B2</i> | 1428 | 89 (83) | 10 (10) | 138 (117) | 6 (6) | 172 |
| <i>CYP11A1</i> | 213 | 52 (52) | 1 (1) | 21 (21) | 43 (43) | 103 |
| <i>CYP17A1</i> | 171 | 24 (24) | NA | 11 (11) | 23 (22) | 42 |
| <i>CYP21A2</i> | 508 | 57 (56) | 11 (10) | 76 (75) | 25 (22) | 142 |
| <i>3βHSDII</i> | 414 | 67 (63) | NA | 26 (26) | 46 (44) | 106 |
| <i>StAR</i> | 624 | 110 (101) | 4 (4) | 139 (128) | 11 (11) | 189 |
| <i>HSD11B1</i> | 343 | 164 (135) | 1 (1) | 50 (44) | 70 (65) | 168 |
| <i>HSD11B2</i> | 550 | 82 (78) | 4 (4) | 53 (50) | 33 (31) | 118 |

The 3'UTR length of the genes involved in corticosteroidogenesis was identified using the UCSC Genome Browser Gateway and Ensemble Genome Browser. The number of predicted miRNA target sites from each database is listed and the number of individual miRNA predicted to bind is shown in parentheses. The final column shows the cumulative number of miRNAs target-sites, with duplicates removed. NA: Not applicable/no predictions.

Table 4-4. Identities of miRNAs with Putative Binding Sites in the *CYP11B1* Gene, but not *CYP11B2*.

| Putative miRNA binding sites in the <i>CYP11B1</i> genes | | | |
|---|-----------------|-----------------|-----------------|
| hsa-miR-105 | hsa-miR-361-3p | hsa-miR-543 | hsa-miR-661 |
| hsa-miR-149* | hsa-miR-371-5p | hsa-miR-545 | hsa-miR-665 |
| hsa-miR-150 | hsa-miR-373 | hsa-miR-548d-3p | hsa-miR-744 |
| hsa-miR-151-5p | hsa-miR-376a | hsa-miR-548o | hsa-miR-768-5p |
| hsa-miR-181a | hsa-miR-376b | hsa-miR-550 | hsa-miR-876-5p |
| hsa-miR-181b | hsa-miR-382 | hsa-miR-551a | hsa-miR-924 |
| hsa-miR-181c | hsa-miR-423-5p | hsa-miR-554 | hsa-miR-933 |
| hsa-miR-181d | hsa-miR-429 | hsa-miR-561 | hsa-miR-938 |
| hsa-miR-190b | hsa-miR-431 | hsa-miR-568 | hsa-miR-1197 |
| hsa-miR-193a-5p | hsa-miR-432 | hsa-miR-583 | hsa-miR-1207-5p |
| hsa-miR-196a | hsa-miR-449a | hsa-miR-588 | hsa-miR-1224-5p |
| hsa-miR-196b | hsa-miR-449b | hsa-miR-590-3p | hsa-miR-1225-5p |
| hsa-miR-200b | hsa-miR-452 | hsa-miR-593 | hsa-miR-1226 |
| hsa-miR-200c | hsa-miR-483-3p | hsa-miR-596 | hsa-miR-1244 |
| hsa-miR-204 | hsa-miR-490-3p | hsa-miR-599 | hsa-miR-1248 |
| hsa-miR-205 | hsa-miR-494 | hsa-miR-608 | hsa-miR-1251 |
| hsa-miR-210 | hsa-miR-497* | hsa-miR-609 | hsa-miR-1256 |
| hsa-miR-214 | hsa-miR-503 | hsa-miR-618 | hsa-miR-1259 |
| hsa-miR-218 | hsa-miR-515-3p | hsa-miR-620 | hsa-miR-1269 |
| hsa-miR-219-1-3p | hsa-miR-516a-3p | hsa-miR-622 | hsa-miR-1270 |
| hsa-miR-219-2-3p | hsa-miR-518d-5p | hsa-miR-626 | hsa-miR-1273 |
| hsa-miR-221* | hsa-miR-518e* | hsa-miR-627 | hsa-miR-1281 |
| hsa-miR-23a | hsa-miR-519a | hsa-miR-630 | hsa-miR-1290 |
| hsa-miR-23b | hsa-miR-519b-5p | hsa-miR-636 | hsa-miR-1294 |
| hsa-miR-297 | hsa-miR-519c-3p | hsa-miR-637 | hsa-miR-1300 |
| hsa-miR-298 | hsa-miR-519c-5p | hsa-miR-643 | hsa-miR-1303 |
| hsa-miR-299-3p | hsa-miR-519e | hsa-miR-652 | hsa-miR-1304 |
| hsa-miR-302c | hsa-miR-526b | hsa-miR-655 | hsa-miR-1321 |
| hsa-miR-340 | hsa-miR-532-5p | hsa-miR-657 | hsa-miR-1322 |
| hsa-miR-34c-5p | hsa-miR-539 | hsa-miR-660 | |

For information regarding miRNA nomenclature please see Section 1.5.2.1

Table 4-5. Identities of miRNAs with Putative Binding Sites in the *CYP11B1* and *CYP11B2* Genes.

| Putative miRNA binding sites in the CYP11B1 and the CYP11B2 genes | | | |
|---|------------------|----------------|-----------------|
| hsa-miR-1 | hsa-miR-378 | hsa-miR-592 | hsa-miR-1184 |
| hsa-miR-10a | hsa-miR-422a | hsa-miR-604 | hsa-miR-1204 |
| hsa-miR-10b | hsa-miR-450b-3p | hsa-miR-613 | hsa-miR-1205 |
| hsa-miR-138 | hsa-miR-484 | hsa-miR-615-5p | hsa-miR-1207-3p |
| hsa-miR-140-3p | hsa-miR-485-5p | hsa-miR-624 | hsa-miR-1224-3p |
| hsa-miR-143 | hsa-miR-486-3p | hsa-miR-625 | hsa-miR-1236 |
| hsa-miR-146b-3p | hsa-miR-504 | hsa-miR-628-5p | hsa-miR-1253 |
| hsa-miR-185 | hsa-miR-509-3-5p | hsa-miR-632 | hsa-miR-1254 |
| hsa-miR-188-3p | hsa-miR-509-5p | hsa-miR-638 | hsa-miR-1260 |
| hsa-miR-198 | hsa-miR-510 | hsa-miR-647 | hsa-miR-1265 |
| hsa-miR-206 | hsa-miR-515-5p | hsa-miR-651 | hsa-miR-1266 |
| hsa-miR-22 | hsa-miR-516b | hsa-miR-671-5p | hsa-miR-1274a |
| hsa-miR-220c | hsa-miR-520a-5p | hsa-miR-708 | hsa-miR-1274b |
| hsa-miR-24 | hsa-miR-520f | hsa-miR-769-3p | hsa-miR-1275 |
| hsa-miR-28-5p | hsa-miR-548a-3p | hsa-miR-873 | hsa-miR-1280 |
| hsa-miR-328 | hsa-miR-548e | hsa-miR-874 | hsa-miR-1285 |
| hsa-miR-339-5p | hsa-miR-548f | hsa-miR-875-3p | hsa-miR-1286 |
| hsa-miR-33b | hsa-miR-549 | hsa-miR-877 | hsa-miR-1287 |
| hsa-miR-34a | hsa-miR-558 | hsa-miR-939 | hsa-miR-1291 |
| hsa-miR-34c-3p | hsa-miR-571 | hsa-miR-940 | hsa-miR-1299 |
| hsa-miR-370 | hsa-miR-577 | hsa-miR-943 | hsa-miR-1308 |

Table 4-6. Identities of miRNAs with Putative Binding Sites in the *CYP11B2* Gene, but not *CYP11B1*.

| Putative miRNA binding sites in the CYP11B2 genes | | | |
|---|-----------------|--------------|-----------------|
| hsa-miR-125a-3p | hsa-miR-362-5p | hsa-miR-606 | hsa-miR-1225-3p |
| hsa-miR-125a-5p | hsa-miR-367* | hsa-miR-612 | hsa-miR-1228 |
| hsa-miR-125b | hsa-miR-378* | hsa-miR-614 | hsa-miR-1229 |
| hsa-miR-134 | hsa-miR-378b | hsa-miR-619 | hsa-miR-1260b |
| hsa-miR-184 | hsa-miR-378c | hsa-miR-624* | hsa-miR-1914 |
| hsa-miR-188-5p | hsa-miR-383 | hsa-miR-631 | hsa-miR-1914* |
| hsa-miR-18b* | hsa-miR-431* | hsa-miR-639 | hsa-miR-1975 |
| hsa-miR-193 | hsa-miR-432* | hsa-miR-650 | hsa-miR-2113 |
| hsa-miR-193a-3p | hsa-miR-449 | hsa-miR-657 | hsa-miR-3065-3p |
| hsa-miR-193b | hsa-miR-452* | hsa-miR-658 | hsa-miR-3125 |
| hsa-miR-217 | hsa-miR-495 | hsa-miR-661 | hsa-miR-3126-5p |
| hsa-miR-224* | hsa-miR-500* | hsa-miR-665 | hsa-miR-3127 |
| hsa-miR-30c-1* | hsa-miR-505* | hsa-miR-711 | hsa-miR-3153 |
| hsa-miR-30c-2* | hsa-miR-512-3p | hsa-miR-720 | hsa-miR-3162 |
| hsa-miR-31 | hsa-miR-513a-5p | hsa-miR-744* | hsa-miR-3176 |
| hsa-miR-326 | hsa-miR-514 | hsa-miR-760 | hsa-miR-3192 |
| hsa-miR-329 | hsa-miR-523 | hsa-miR-766 | hsa-miR-4292 |
| hsa-miR-337-3p | hsa-miR-525-5p | hsa-miR-801 | hsa-miR-4306 |
| hsa-miR-33a | hsa-miR-552 | hsa-miR-888* | hsa-miR-4313 |
| hsa-miR-346 | hsa-miR-593 | hsa-miR-93* | hsa-miR-4319 |
| hsa-miR-34c | hsa-miR-597 | hsa-miR-942 | hsa-miR-4323 |
| hsa-miR-362-3p | hsa-miR-605 | hsa-miR-1202 | hsa-miR-4327 |

For information regarding miRNA nomenclature please see Section 1.5.2.1

4.4.4 miRNA Expression Profiling of the Adrenal Gland

RNA extracted from four normal human adrenal glands was tested for quality prior to performing the microarray analysis. The quantity of the samples was first assessed using the Nanodrop (Section 2.2.4) then each sample was diluted to run on an Agilent Bioanalyser chip (Section 2.2.5). The electropherogram and RIN (RNA integrity number) are shown in Figure 4-4. The peaks labelled 18S and 28S represent the ribosomal RNA and are used to calculate the RIN. The first small peak is a spike-in RNA control, the second represents small RNA molecules that would encompass miRNAs. The 'Normal Adrenal 4' sample had the lowest RIN which may have proved problematic for mRNA studies, but it had a strong 'microRNA' peak. All samples were subject to further quality control testing by LC Sciences (Houston, Texas) prior to being run on the miRNA microarray. All samples successfully passed the internal quality control tests and, therefore, the miRNA microarray was performed on four normal adrenal gland samples.

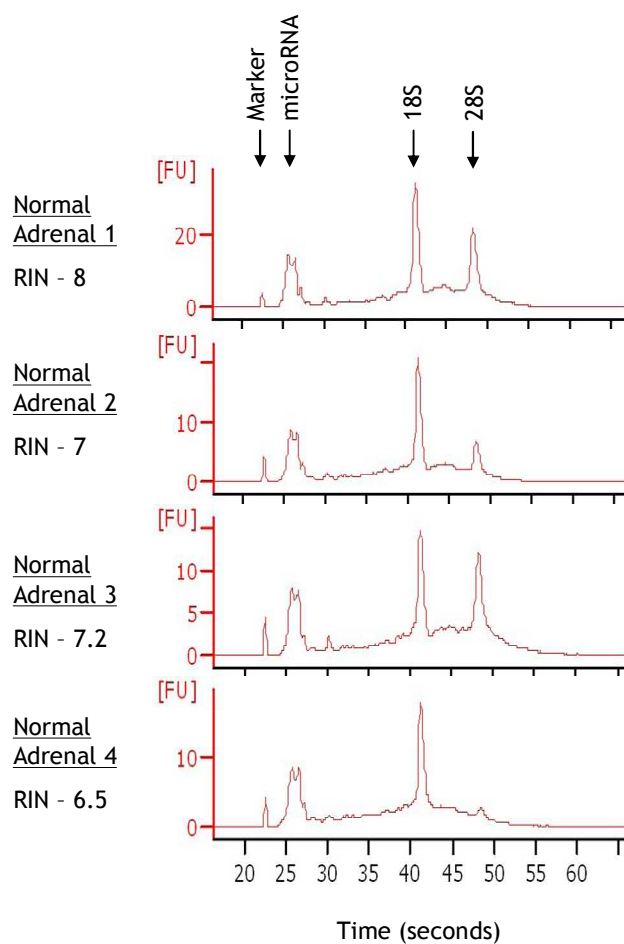


Figure 4-4. Agilent Bioanalyser Electropherogram of Adrenal RNA Samples.
RIN: RNA integrity Number; FU: fluorescence.

The mean microarray output intensity is shown in Figure 4-5 for each of the 728 human miRNAs on the chip (Version 10.1). The manufacturers of the microarray microfluidic chip recommended a cut off value of 500 arbitrary units (AU) to positively determine a miRNA expressed within a sample; this is represented by the red line on Figure 4-5. For future investigations miRNAs expressed below the threshold were discarded, leaving 103 miRNAs expressed in the human adrenal gland above the stated threshold. The mean expression level of these miRNAs is shown in Figure 4-6. The microarray results for individual miRNAs was relatively consistent across each of the four adrenal samples. The expression ranged from 510 ± 88 AU for miR-106a, to the highly expressed miR-26a, expressed at $31,535 \pm 2,012$ AU. Some of the expressed miRNAs included both strands of the precursor-miRNA e.g. pre-miR-574 and pre-miR-768. In this case the -5p and -3p miRNA were expressed at approximately equal levels. However, for pre-miR-151 the -5p miRNA was expressed at a higher level than the -3p form, $6,052 \pm 190$ AU and 943 ± 58 AU respectively. In contrast the expression of miR-199a-5p was (713 ± 135 AU) the miR-199a-3p ($8,405 \pm 1,282$ AU). Eight members of the let-7 (a-g and i) family were highly expressed in the tissue.

To validate the miRNA microarray result, 40 miRNAs were chosen to be quantified by Taqman[®] qRT-PCR. The mean cycle threshold value for each miRNA is shown in Table 4-7. Again, the intra-sample variation was low confirming the homology of the adrenal samples tested. The qRT-PCR results were normalised to a housekeeping gene, RNU48, creating a delta Ct value (ΔCt). The negative ΔCt was expressed to the power of 2 (representing the doubling of RNA concentration per Ct). This value is, therefore, proportional to the abundance of the miRNA in the sample. These results were highly and significantly correlated with the microarray values; $r^2 = 0.50$ and $p < 0.0001$ (Figure 4-7). One notable outlier was miR-638, which had a relatively high expression as quantified by microarray analysis ($17,630 \pm 1,681$ AU), but low expression according to qRT-PCR validation (mean Ct value 29.78 ± 0.18 ; $2^{-\Delta Ct}$ equivalent: 0.004) (Figure 4-7 and Table 4-7). qRT-PCR is the gold standard for expression quantification and therefore miR-638 should be regarded as a low-expression miRNA in the adrenal gland. Other than this the qRT-PCR results confirmed the findings of the miRNA microarray experiment.

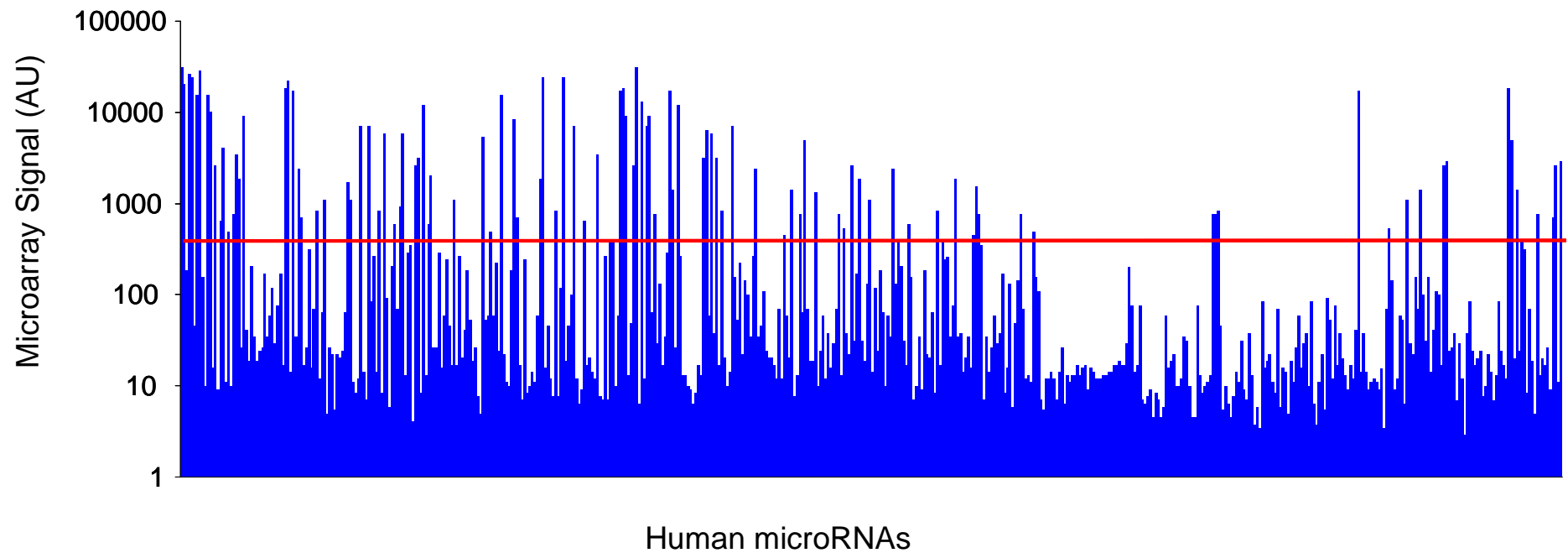


Figure 4-5. Normal Adrenal Gland miRNA Microarray Output.

The average normalised microarray expression signal of 728 human miRNAs (x-axis, in miRNA numerical order) in four normal adrenal glands. The red line represents the expression cut-off threshold of 500 Arbitrary Units (AU).

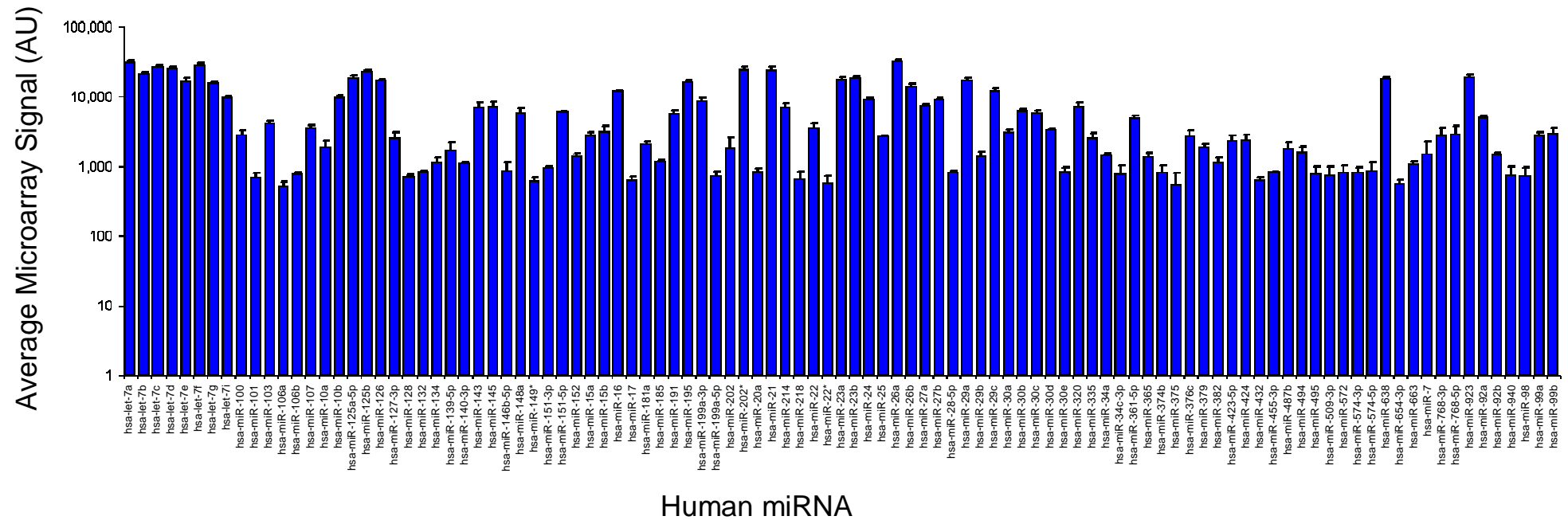


Figure 4-6. Average Microarray Signals of miRNAs Expressed in the Adrenal Gland.

The average normalised microarray expression signal of miRNAs with an expression level greater than 500 Arbitrary Units (AU). The mean of four tissues, error bars represent SEM.

Table 4-7. qRT-PCR Expression Validation of Selected miRNAs.

| miRNA Taqman® Assay | Ct value | Standard Error | miRNA Taqman® Assay | Ct value | Standard Error |
|---------------------|----------|----------------|---------------------|----------|----------------|
| hsa-let-7a | 20.75 | 0.12 | hsa-miR-185 | 31.35 | 0.34 |
| hsa-let-7b | 20.63 | 0.11 | hsa-miR-195 | 21.42 | 0.12 |
| hsa-let-7e | 25.62 | 0.15 | hsa-miR-202 | 25.74 | 0.10 |
| hsa-miR-101 | 25.10 | 0.13 | hsa-miR-214 | 24.08 | 0.13 |
| hsa-miR-103 | 23.05 | 0.08 | hsa-miR-218 | 25.25 | 0.19 |
| hsa-miR-106a | 25.76 | 0.09 | hsa-miR-22 | 22.74 | 0.06 |
| hsa-miR-106b | 24.39 | 0.08 | hsa-miR-23a | 23.43 | 0.13 |
| hsa-miR-107 | 27.35 | 0.06 | hsa-miR-23b | 22.58 | 0.11 |
| hsa-miR-10a | 24.44 | 0.10 | hsa-miR-24 | 21.60 | 0.09 |
| hsa-miR-10b | 22.50 | 0.17 | hsa-miR-26b | 21.22 | 0.07 |
| hsa-miR-125b | 20.33 | 0.10 | hsa-miR-29b | 23.16 | 0.12 |
| hsa-miR-126 | 21.38 | 0.14 | hsa-miR-30b | 21.22 | 0.09 |
| hsa-miR-132 | 25.19 | 0.12 | hsa-miR-30c | 21.68 | 0.08 |
| hsa-miR-134 | 25.99 | 0.12 | hsa-miR-320 | 22.72 | 0.08 |
| hsa-miR-143 | 24.45 | 0.24 | hsa-miR-34a | 23.70 | 0.17 |
| hsa-miR-145 | 24.25 | 0.18 | hsa-miR-379 | 25.38 | 0.07 |
| hsa-miR-150 | 25.71 | 0.36 | hsa-miR-382 | 25.90 | 0.14 |
| hsa-miR-15a | 25.57 | 0.12 | hsa-miR-432 | 26.11 | 0.15 |
| hsa-miR-16 | 20.58 | 0.13 | hsa-miR-487b | 24.62 | 0.14 |
| hsa-miR-181a | 24.97 | 0.16 | hsa-miR-638 | 29.78 | 0.18 |

Mean cycle threshold (Ct) values are from four adrenal gland samples measured by qRT-PCR in technical triplicate (three repeats per sample per assay)

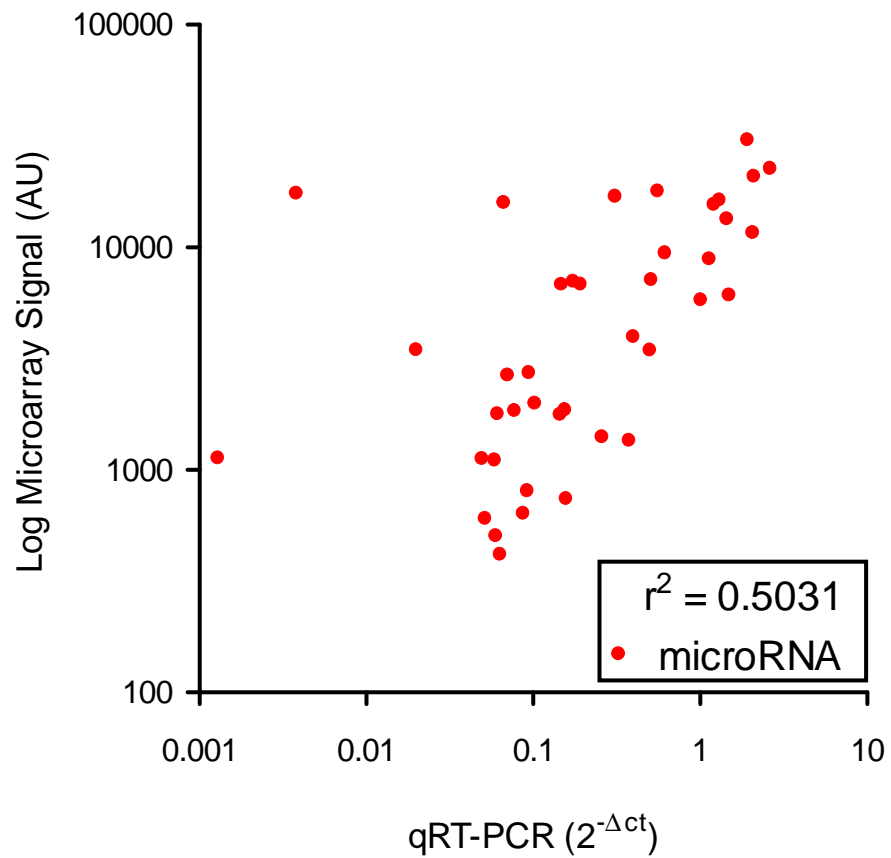


Figure 4-7. Correlation of Microarray and qRT-PCR miRNA Expression.

The microarray expression levels of twenty miRNAs plotted against their qRT-PCR expression. The cycle threshold (Ct) the housekeeping reference, RNU48, was deducted from that of the miRNA (ΔCt). The negative ΔCt was expressed to the power of 2 (assuming 100% efficiency).

4.4.5 Adrenal-miRNAs with *CYP11B1* and *CYP11B2* Binding Sites

To identify the miRNAs which may regulate *CYP11B1* and *CYP11B2* mRNA in the adrenal gland, the putative miRNA-target sites and miRNA expression data sets were combined. The results of this are summarised in Figure 4-8. Half of the miRNA target sites for *CYP11B1* are shared with the target sites predicted for *CYP11B2*, whereas, the miRNAs predicted to target both genes comprise the majority of these predicted to target *CYP11B2*. In total, 24 adrenal-expressed miRNAs were predicted to bind *CYP11B1* 3'UTR and 16 to bind *CYP11B2* 3'UTR; in total, 28 miRNAs are predicted to bind to both genes. Therefore, based on the computational approaches used, 75 miRNAs expressed in the adrenal gland do not bind to the *CYP11B1* or *CYP11B2* mRNA.

Detailed information for the 28 miRNAs is shown in Table 4-8. None of these miRNAs are located in genes which have been directly implicated in corticosteroidogenesis. However, their genetic locations may prove interesting: both miR-638 and miR-214 are located in a region coding for dynamin mRNA (type 2 and 3 respectively); miR-218 and miR148* are located in the *SLIT2* gene and the *GPC1* gene, whose products are known to interact; miR-10a and miR-10b are both located in genes belonging to the homeobox transcription factor family. Finally, several miRNAs are transcribed in clusters including miR-24 and miR-23b which are in the *C9orf3* cluster.

Table 4-9 and Table 4-10 give specific information regarding the bioinformatic target site predictions, including which database(s) predicted each miRNA binding site, the number of binding sites predicted and the proposed degree of base-pairing complementarity in the seed region (Figure 4-1). These tables also contain the microarray expression values for each miRNA (Table 4-9 and Table 4-10). Only three of the miRNAs (miR-149*, miR-423-5p and miR-34a) were predicted to possess two separate target sites, for miR-149* and miR-423-5 the binding sites are relatively close, (206 and 69 bases apart, respectively). Most target site predictions were provided by only one or two of the databases but three databases (the exception being miRViewer) predict a binding site for miR-638 on the *CYP11B1* 3'UTR (Table 4-9).

Given the tendency of bioinformatics databases to rely on seed site base-pairing for miRNA-target prediction, it is unsurprising that the majority of target sites predicted have full complementarity at the seed region (Figure 4-9) and that most are 7mer predictions, indicating a strong likelihood of miRNA regulation. miR-940 is predicted to bind with the strongest seed-pairing, 8mer, to both of the genes; miR-149* is also predicted to have an 8mer site on the *CYP11B1* gene (Table 4-9). Two miRNAs, miR-125a-5p and miR-125b, are predicted to bind with a 8mer seed site to the *CYP11B2* gene (Table 4-10). These miRNAs belong to the same miRNA family and therefore share a high degree of sequence similarity with identical sequence in the seed region; for this reason their 8mer predicted site lies in exactly the same place on the 3'UTR.

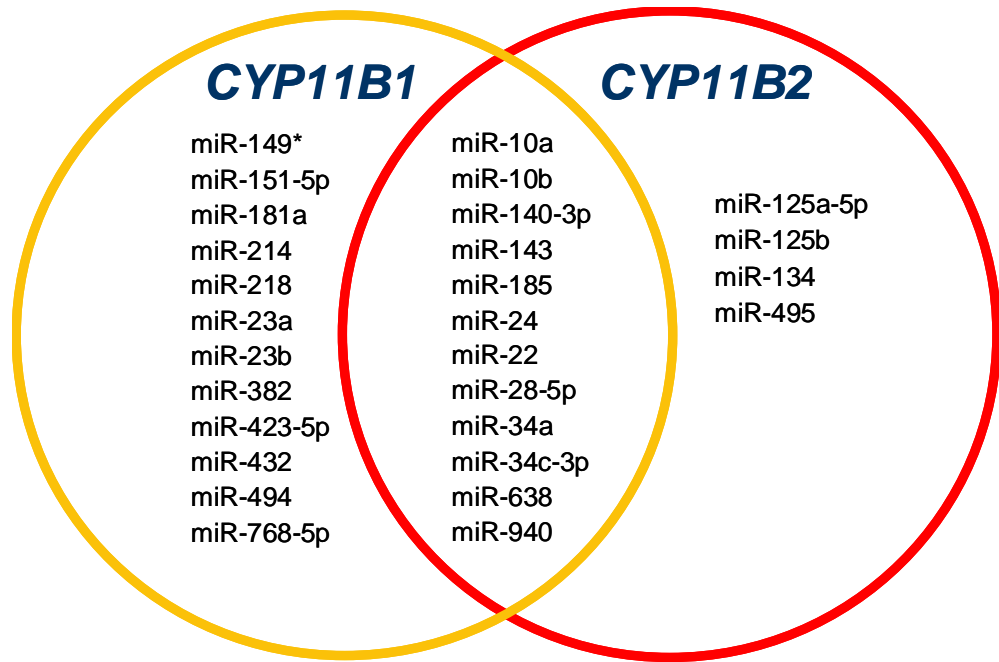


Figure 4-8. Venn Diagram of Adrenal miRNAs with Putative miRNA-Target Sites in the 3'UTR of the *CYP11B1* and *CYP11B2* Genes

miRNA microarray analyses of non-tumorous adrenal tissue combined with bioinformatics analysis of putative miRNA binding sites in the 3'-UTR of the *CYP11B1* and *CYP11B2* genes

Table 4-8. Genomic and Sequence Information of Adrenal-miRNAs Predicted to Bind to *CYP11B1* and *CYP11B2*.

| microRNA | miRNA Sequence | Genetic Location | Co-ordinates of miRNA Location |
|----------------|---------------------------|------------------|------------------------------------|
| hsa-miR-149* | aggaggacggggcugugc | Intronic | 2 : 241,395,418 - 241,395,506 [+] |
| hsa-miR-151-5p | ucgaggagcucacagucuagu | Intronic | 8 : 141,742,663 - 141,742,752 [-] |
| hsa-miR-181a | aacauucaacgcugucggugagu | Intergenic | 1 : 198,828,173 - 198,828,282 [-] |
| hsa-miR-214 | acagcaggcacagacaggcagu | Intronic | 1 : 172,107,938 - 172,108,047 [-] |
| hsa-miR-218 | uugugcuugaucuaaccaugu | Intronic | 4 : 20,529,898 - 20,530,007 [+] |
| hsa-miR-23a | aucacauugccagggauuucc | Intergenic | 19 : 13,947,401 - 13,947,473 [-] |
| hsa-miR-23b | aucacauugccagggauuacc | Intronic | 9 : 97,847,490 - 97,847,586 [+] |
| hsa-miR-382 | gaaguuguucgugggguuucg | Intergenic | 14 : 101,520,643 - 101,520,718 [+] |
| hsa-miR-423-5p | ugaggggcagagagcgagacuuu | Intronic | 17 : 28,444,097 - 28,444,190 [+] |
| hsa-miR-432 | ucuuggaguaggucuuugggugg | Intergenic | 14 : 101,350,820 - 101,350,913 [+] |
| hsa-miR-494 | ucuuggaguaggucuuugggugg | Intergenic | 14 : 101,495,971 - 101,496,051 [+] |
| hsa-miR-768-5p | guuggaggauaaguuacggagugau | Intergenic | 16 : 70,349,796 - 70,349,899 [-] |
| hsa-miR-10a | caauucguaucuaggggaaua | Intronic | 17 : 46,657,200 - 46,657,309 [-] |
| hsa-miR-10b | uaccugugaagcgaauuugug | Intronic | 2 : 177,015,031 - 177,015,140 [+] |
| hsa-miR-140-3p | cagugguuuacccuauuguag | Intronic | 16 : 69,966,984 - 69,967,083 [+] |
| hsa-miR-143 | ugagaugaagcacuguagcuc | Intergenic | 5 : 148,808,481 - 148,808,586 [+] |
| hsa-miR-185 | uggagagaaaggcaguuccuga | Intronic | 22 : 20,020,662 - 20,020,743 [+] |
| hsa-miR-22 | aagcugccaguugaagaacugu | Intronic | 17 : 1,617,197 - 1,617,281 [-] |
| hsa-miR-24 | uggcucaguucagcaggaacag | Intronic | 9 : 97,848,303 - 97,848,370 [+] |
| hsa-miR-28-5p | aaggagcucacagucuuugag | Intronic | 3 : 188,406,569 - 188,406,654 [+] |
| hsa-miR-34a | uggcagugucuagcugguugu | Intergenic | 1 : 9,211,727 - 9,211,836 [-] |
| hsa-miR-34c-3p | aaucacuaaccacacggccagg | Intergenic | 11 : 111,384,164 - 111,384,240 [+] |
| hsa-miR-638 | agggaucgcggcggguggcgccu | Intronic | 19 : 10,829,080 - 10,829,179 [+] |
| hsa-miR-940 | aaggcaggccccgcucccc | Intergenic | 16 : 2,321,748 - 2,321,841 [+] |
| miR-125a-5p | ucccugagaccuuuaaccuguga | Intergenic | 19 : 52,196,507 - 52,196,592 [+] |
| miR-125b | ucccugagaccuaacuuguga | Intergenic | 11 : 121,970,465 - 121,970,552 [-] |
| miR-134 | ugugacugguugaccagagggg | Intergenic | 14 : 101,521,024 - 101,521,096 [+] |
| miR-495 | aaacaaacauuggucacuucuu | Intergenic | 14 : 101,500,092 - 101,500,173 [+] |

Adrenal miRNA information obtained from miRBASE. miRNA sequence: 5'- 3' direction. Genomic co-ordinates: chromosome number: co-ordinates [strand]. The double lines separate miRNAs according to bioinformatic prediction, the top section includes miRNAs with putative binding site in *CYP11B1*, the middle section miRNAs predicted to bind to both genes; and the bottom miRNAs putative binding sites on the *CYP11B2* 3'UTR.

Table 4-9. Bioinformatic and Microarray Information of Adrenal-miRNA with Putative Binding Sites in the *CYP11B1* 3'UTR.

| microRNA | Number of Database Hits | Database | | | | Number of Binding Sites | Seed Site Match | Distance Between Binding Sites (nucleotides) | Microarray Average Signal Intensity (AU) | |
|----------------|-------------------------|------------|--------------|-----------|-----------|-------------------------|---------------------------|--|--|-------|
| | | TargetScan | microRNA.org | miRViewer | MicroCosm | | | | | |
| miR-149* | 1 | | | | ✓ | 2 | 8mer / Offset 6mer | 206 | 608 ± | 89 |
| miR-151-5p | 2 | ✓ | | | ✓ | 1 | 7mer-m8 | | 6,052 ± | 190 |
| miR-181a | 1 | ✓ | | | | 1 | 7mer-m8 | | 2,011 ± | 274 |
| miR-214 | 1 | | ✓ | | | 1 | Offset 6mer | | 6,894 ± | 1,106 |
| miR-218 | 1 | ✓ | | | | 1 | 7mer-1A | | 642 ± | 197 |
| miR-23a | 2 | ✓ | ✓ | | | 1 | 7mer-m8 | | 17,047 ± | 2,074 |
| miR-23b | 2 | ✓ | ✓ | | | 1 | 7mer-m8 | | 18,033 ± | 1,998 |
| miR-382 | 1 | | | | ✓ | 1 | Offset 6mer | | 1,116 ± | 195 |
| miR-423-5p | 2 | ✓ | ✓ | | | 2 | Offset 6mer / Offset 6mer | 69 | 2,272 ± | 507 |
| miR-432 | 2 | | ✓ | | ✓ | 1 | Offset 6mer | | 611 ± | 90 |
| miR-494 | 1 | ✓ | | | | 1 | 7mer-1A | | 1,576 ± | 369 |
| miR-768-5p | 1 | ✓ | | | | 1 | 7mer-1A | | 2,832 ± | 1,021 |
| hsa-miR-10a | 2 | ✓ | | | ✓ | 1 | 7mer-1A | | 1,872 ± | 470 |
| hsa-miR-10b | 2 | ✓ | | | ✓ | 1 | 7mer-1A | | 9,496 ± | 1,152 |
| hsa-miR-140-3p | 1 | | ✓ | | | 1 | 7mer-m8 | | 1,082 ± | 79 |
| hsa-miR-143 | 1 | ✓ | | | | 1 | 7mer-1A | | 6,877 ± | 1,236 |
| hsa-miR-185 | 1 | ✓ | | | | 1 | 7mer-m8 | | 1,141 ± | 90 |
| hsa-miR-22 | 1 | ✓ | | | | 1 | 7mer-m8 | | 3,473 ± | 670 |
| hsa-miR-24 | 2 | | ✓ | ✓ | | 2 | Offset 6mer | 930 | 8,938 ± | 810 |
| hsa-miR-28-5p | 2 | ✓ | ✓ | | | 1 | 7mer-m8 | | 788 ± | 75 |
| hsa-miR-34a | 2 | ✓ | ✓ | | | 2 | 7mer-m8/Offset 6mer | 1,275 | 1,418 ± | 98 |
| hsa-miR-34c-3p | 1 | ✓ | | | | 1 | 7mer-1A | | 752 ± | 264 |
| hsa-miR-638 | 3 | ✓ | ✓ | | ✓ | 1 | 7mer-1A | | 17,630 ± | 1,681 |
| hsa-miR-940 | 2 | ✓ | ✓ | | | 1 | 8mer | | 735 ± | 264 |

Table 4-10. Bioinformatic and Microarray Information of Adrenal-miRNA with Putative Binding Sites in the *CYP11B2* 3'UTR.

| microRNA | Number of Database Hits | Database | | | | Number of Binding Sites | Seed Site Match | Distance Between Binding Sites (nucleotides) | Microarray Average Signal Intensity (AU) | |
|-----------------|-------------------------|------------|--------------|-----------|-----------|-------------------------|---------------------|--|--|---------|
| | | TargetScan | microRNA.org | miRViewer | MicroCosm | | | | | |
| hsa-miR-125a-5p | 2 | ✓ | ✓ | | | 1 | 8mer | | 18,278 | ± 1,808 |
| hsa-miR-125b | 2 | ✓ | ✓ | | | 1 | 8mer | | 22,781 | ± 1,558 |
| hsa-miR-134 | 1 | ✓ | | | | 1 | 7mer-m8 | | 1,130 | ± 217 |
| hsa-miR-495 | 1 | ✓ | | | | 1 | 7mer-m8 | | 777 | ± 210 |
| hsa-miR-10a | 1 | ✓ | | | | 1 | 7mer-1A | | 1,872 | ± 470 |
| hsa-miR-10b | 2 | ✓ | | | ✓ | 1 | 7mer-1A | | 9,496 | ± 1,152 |
| hsa-miR-140-3p | 1 | ✓ | | | | 1 | 7mer-m8 | | 1,082 | ± 79 |
| hsa-miR-143 | 1 | ✓ | | | | 1 | 7mer-1A | | 6,877 | ± 1,236 |
| hsa-miR-185 | 2 | ✓ | ✓ | | | 1 | 7mer-m8 | | 1,141 | ± 90 |
| hsa-miR-22 | 2 | ✓ | | ✓ | | 1 | 7mer-m8 | | 3,473 | ± 670 |
| hsa-miR-24 | 1 | | | ✓ | | 1 | Offset 6mer | | 8,938 | ± 810 |
| hsa-miR-28-5p | 2 | ✓ | ✓ | | | 1 | 7mer-m8 | | 788 | ± 75 |
| hsa-miR-34a | 1 | | | ✓ | | 2 | 7mer-m8/Offset 6mer | 1,275 | 1,418 | ± 98 |
| hsa-miR-34c-3p | 1 | ✓ | | | | 1 | 7mer-1A | | 752 | ± 264 |
| hsa-miR-638 | 1 | ✓ | | | | 1 | 7mer-1A | | 17,630 | ± 1,681 |
| hsa-miR-940 | 2 | ✓ | ✓ | | | 1 | 8mer | | 735 | ± 264 |

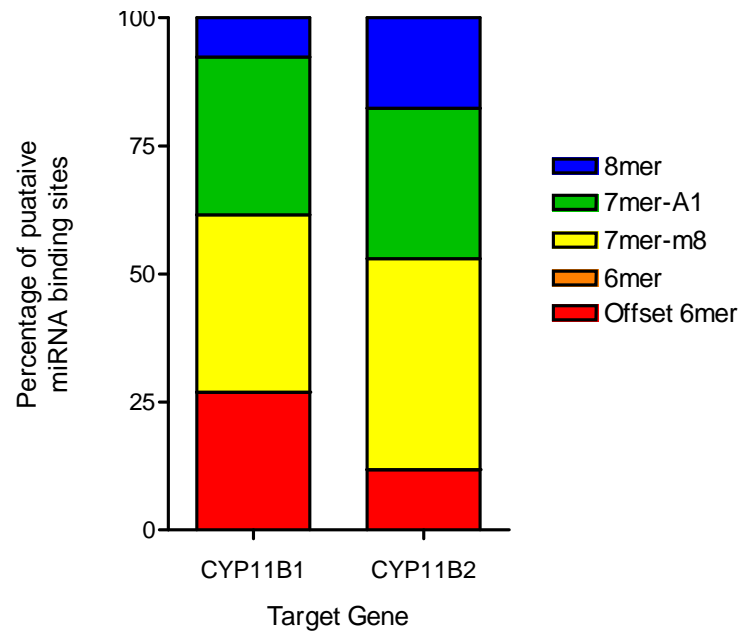


Figure 4-9. Seed Site Distribution of Putative miRNA Binding Sites.

Base-pairing in the seed region of miRNAs with putative binding sites in the *CYP11B1* and *CYP11B2* 3'UTR.

4.5 Discussion

The experiments presented in this chapter investigated the existence of miRNA target sites in corticosteroidogenic genes using computational methods. It also identified the array of miRNAs expressed within the adrenal gland: this data was used to construct a list of potential miRNAs which may regulate the *CYP11B1* and *CYP11B2* genes *in vivo*. The main outcome of this part of the project was the identification of 24 miRNAs expressed in the adrenal gland which may regulate *CYP11B1* transcription and 16 that may regulate *CYP11B2*.

The first stage of *in silico* analysis investigated whether any non-coding RNAs are located within the coding regions of genes involved in corticosteroidogenesis. None of the 1,048 miRNAs currently registered at the miRNA repository, MiRBase (Chobanian *et al.*, 2003;Griffiths-Jones *et al.*, 2006;Griffiths-Jones *et al.*, 2008) were mapped to genomic regions encompassing the important corticosteroidogenic genes, including *CYP11B1* and *CYP11B2*. Hence, altered transcriptional regulation of these genes or SNPs located in these genes will not directly affect the transcription or quantity of any miRNA. Therefore, miRNAs found to regulate *CYP11B1* and *CYP11B2* will be located in separate genomic locations and subject to their own transcriptional regulation.

In-depth analysis of the 3'UTR sequence of *CYP11B1* and *CYP11B2* revealed both to be conserved across a few, but not all, mammalian species. Conservation of the 3'UTR and, in particular the putative miRNA-target sites is proposed to be an indicator of miRNA regulation (Friedman *et al.*, 2009). miRNA sequences themselves are highly conserved, yet there are examples of mammalian-specific miRNAs which may target non-conserved sites (Sethupathy *et al.*, 2006;Friedman *et al.*, 2009). It could be argued that target site conservation is preferentially selected for or that non-conserved regions in higher mammals have evolved. The *CYP11B1* and *CYP11B2* genes have the longest 3'UTRs of all the genes analysed and this might reflect the importance of this region for regulation of mRNA stability. Studies have shown that genes involved in basic cell homeostasis, for example ribosomal genes, have shorter 3'UTRs and are resistant to miRNA-mediated repression (Stark *et al.*, 2005). Assessment of the unbound secondary mRNA structure was performed but without the addition of a miRNA,

it is difficult to interpret this. The software used to generate this structure cannot generate miRNA interactions but an alternative software package, RNAHybrid, has been developed (Kruger and Rehmsmeier, 2006). This software will be useful for assessing the impact of miRNAs on the secondary structure of the *CYP11B1* or *CYP11B2* 3'UTR and evaluating the minimum free energy of binding (Kruger and Rehmsmeier, 2006) and will be utilised in Section 5.4.9.

Another parameter which may influence miRNA regulation is the turnover rate of mRNA, which is determined by the rate of transcription vs. the rate of mRNA degradation. mRNAs with a relatively quick turnover are less likely to be subject to miRNA regulation and more likely to be regulated by other degrading factors that act on AU-rich element (AREs). However, those with longer half-lives are more likely to be regulated by miRNAs (Larsson *et al.*, 2010). The assessment of the half-lives of *CYP11B1* and *CYP11B2* mRNA has been previously estimated in H295R cells. These were treated with Actinomycin D for 2 hr, then the relative abundance of the mRNA transcripts measured at hourly intervals (Lin *et al.*, 2006). The abundance of both *CYP11B1* and *CYP11B2* mRNA decreased to half of the starting level by approximately 3 hr. This is on the borderline of what Larsson *et al.* (2010) describe as a slow or medium turnover rate and, based on their analysis, *CYP11B1* and *CYP11B2* mRNA are less likely to be regulated by miRNAs than a longer turnover rate mRNA (half-life > 1000 minutes). However, they did show that 4.4% of the slower turnover mRNAs were liable to strong repression by miRNAs, compared to 10.2% of long turnover mRNAs. There is also quite a lot of scatter within their data; 3 of the previously published data-sets used did not show a correlation of repression to turnover rate. Finally, the data-sets were all generated in a non-specific cell line (HeLa), used miRNA species and defined miRNA-mediated regulation by strong repression of mRNA target (Larsson *et al.*, 2010) which may not reflect canonical fine-tuning miRNA action in specific cell types (Sevignani *et al.*, 2006). The authors also mention that the relative abundance of mRNA could have an impact on regulation. Thus, in the *CYP11B1* and *CYP11B2* expression-specific cells of the adrenal cortex, this may not be a factor. The importance of mRNA turnover rate remains to be seen.

miRNA target sites were predicted by bioinformatic analysis all of the genes analysed. Of the databases employed, only Tarbase failed to predict any miRNA target sites, presumably because it relies solely on experimentally-validated

miRNA targets (Papadopoulos *et al.*, 2009), and there are currently no validated miRNA, mRNA targets in this pathway (Section 1.9).

In general, there was a large degree of variation in the number of predicted sites generated by the other four databases. miR-viewer predicted the lowest number of miRNA binding sites and, for *CYP17A1* and *3BHSDII* mRNA, it failed to predict any sites at all. The database with the next lowest prediction count was MicroCosm Targets. The algorithms used by the TargetScan and microrna.org databases appear to have equivalent stringencies for identifying miRNA target sites, as demonstrated by their similar number of target-site predictions. Interestingly, both of these databases have user-definable parameters, e.g. the levels of miRNA sequence conservation. The results presented from these two databases in this thesis have been generated without restrictions on conservation. In fact, TargetScan identified no miRNA-target sites for evolutionarily conserved miRNAs in the *CYP11B1* or *CYP11B2* 3'UTRs. The exact details of what governs miRNA:mRNA target interaction are still being investigated and, as such, databases are continually evolving. Therefore, it was decided not to increase the stringency of conservation required for miRNA-target prediction, thus avoiding excessive false negative predictions, and also to use a combinational approach of several databases to improve accuracy.

The degree of variation between the databases is consistent with other reports (Bartel, 2009; Saito and Saetrom, 2010). This highlights the current lack of understanding regarding miRNA-target binding and the fact that relatively few miRNA targets have been experimentally validated. Two recent reviews on this subject highlighted the variation between the current group of bioinformatic databases and emphasised that validation is biased towards complementary seed site binding and sequence conservation (Bartel, 2009; Saito and Saetrom, 2010). However, this may itself be biased due to the high weighting placed on these features by several of the commonly-used databases. Other features which are absent from the currently-available databases include alternative miRNA:mRNA complementarity (e.g. centered site base-pairing, Figure 4-1); accounting for optimal distance between multiple miRNA binding sites; or the ability to search for binding sites in the coding-region or 5' untranslated region of genes (Saito and Saetrom, 2010).

Based on computational bioinformatics it appears that all the genes within the corticosteroidogenic pathway possess miRNA binding sites and hence could be subject to miRNA-mediated regulation. As previously stated, the main focus of this thesis is the regulation of *CYP11B1* and *CYP11B2* mRNA expression, and therefore the remainder of the work presented will be concerned with these genes. Bioinformatic prediction identified 202 and 172 miRNA binding sites in the 3'UTRs of the *CYP11B1* and *CYP11B2* genes, respectively. There was a relatively high overlap of miRNA binding-site predictions; given the high degree of sequence similarity in the 3'UTRs (approximately 80%) this is probably not surprising. Moreover, it is comparable to the similarity observed in transcription factor binding sites located at the 5'UTR (Bassett *et al.*, 2000; Bassett *et al.*, 2002). However, there are numerous miRNAs with putative binding sites in only one of the genes so, potentially, miRNA-mediated regulation could be achieved by target-sites common to both genes or by specific miRNAs, thereby accounting for the differential regulation of these genes.

Two striking observations arise from the predictions: the lack of any let-7 miRNA family target-sites and the high number of miRNA* (passenger strand) predictions for the *CYP11B2* 3'UTR. The let-7 family includes the let-7c miRNA, which was the second miRNA to be identified (Reinhart *et al.*, 2000). This family is believed to have a major role in developmental timing and cell regulation (Roush and Slack, 2008) and hence would be thought unlikely to target the *CYP11B1* and *CYP11B2* genes. miRNA* are thought to be the redundant strand of the pre-miR hair-pin that generates the functioning miRNA (Section 1.5.2.1 and 1.5.2.3). However, as the function of more miRNAs is being discovered, many are being renamed to indicate from which arm they are cleaved (-3p or -5p) and, as such, do not indicate which mature arm of the pre-miRNA is likely to be functional. Therefore, the miRNA* predictions for *CYP11B2* should not be discounted as regulators and, in fact, regulation of *CYP11B2* may even represent a novel function of these * miRNAs.

To determine the miRNA expression profile of the adrenal gland, human non-tumorous (termed 'normal') adrenal glands were chosen for miRNA microarray analysis. The samples were obtained from four kidney donor patients undergoing nephrectomy and their adrenal glands were retained and frozen at -80°C. RNA isolation from the tissues was successful, each sample obtaining strong RIN

scores. The microarray and subsequent qRT-PCR validation indicated that the samples were of good quality and homogeneous for miRNA expression. Therefore, the results in this thesis can confidently confirm the expression of 103 human miRNAs in the adrenal gland. At the time the miRNA microarray was performed it contained probes which covered 100% of the known human miRNAs and therefore, represented the best option for assessing the relative levels of all miRNA in samples. The quality of the microarray was confirmed by qRT-PCR performed in the same samples; this correlated highly with the microarray expression results.

The samples of adrenal gland used for RNA isolation could not be cut precisely enough to include only tissue from the adrenal cortex and may also have included part of the adrenal medulla. Moreover, they would likely have contained portions of all three layers of the adrenal cortex. Therefore, the miRNA expression profile generated in this thesis is representative of the whole adrenal gland. It would be beneficial to obtain tissue from specific zones of the cortex in order to determine the zone-specific distribution, if any, of individual miRNAs. However, obtaining sufficient human, normal adrenal tissue for this has, as of yet, proved difficult.

When this investigation started, there were no existing publications describing adrenal miRNA expression. However, there have since been a small number of such studies with the main focus being on adrenal pathology *e.g.* adrenocortical adenoma or carcinoma. These have not studied normal adrenal function or the role of miRNAs in hypertension and have only used normal adrenal glands for purposes of comparison (Tombol *et al.*, 2009; Soon *et al.*, 2009; Iliopoulos *et al.*, 2009; Bimpaki *et al.*, 2010; Schmitz *et al.*, 2011). In general, it is hard to compare the miRNA-expression profiling in these publications with that which is presented in this thesis. It appears that none of the publications have submitted their microarray results to a microarray repository to allow open access of individual miRNA expression results, meaning that only information regarding selected published miRNAs can be obtained. Furthermore, comparison is complicated as all of these studies used different profiling platforms to each other, and to the one used in this thesis. Only the study by Soon *et al.*, (2009) used microarray system of the same level of miRNA coverage as this study (100% of miRNAs in miRBase v10). The others used a Taqman Low Density Array® (Applied

Biosciences) platform which is a good alternative for assessing miRNA expression but typically contains fewer miRNA probes (365 probes) than chip-based arrays. Finally, interpreting the data is complicated due to post-assay data manipulation for example depicting expression levels by hierarchical heat-map clustering, meaning that relative quantities of miRNAs can not be compared. However, careful analysis shows that at least 33 of the 103 miRNAs identified in this thesis have been previously identified (Tombol *et al.*, 2009; Soon *et al.*, 2009; Iliopoulos *et al.*, 2009; Bimpaki *et al.*, 2010; Schmitz *et al.*, 2011). This number may in fact be higher but full data-sets are not available and, for one study (Schmitz *et al.*, 2011), the online supplementary data is not currently accessible and another presents only profiling data from tumorous tissue (Soon *et al.*, 2009). Several miRNAs have been consistently detected both in the current study and in more than one of the other published studies: miR-100, miR-143, miR-145, miR-375, miR-424 and miR-7. Without access to the full data from the published microarray studies it is impossible to comment on how representative is the data presented in this thesis, but even the small degree of overlap described above suggests that the findings in this thesis are comparable. This current study presents the most comprehensive adrenal miRNA expression investigation in normal subjects; with the exception of Tombol *et al.*, (2009), the other studies used either commercially-purchased, pooled adrenal samples or adjacent adrenal tissue lying next to an adrenal tumour. Tombol *et al.*, (2009) used adrenal tissue samples obtained from patients undergoing nephrectomy for kidney tumours and, while there is no reason to suggest that the adrenal glands were also affected by tumorous tissue, the adrenal gland and kidney are linked by blood supply and miRNAs can circulate through the bloodstream (Section 1.8.2). Therefore, using the adrenal glands of kidney donor patients, as in the current study, is probably the most reliable source of normal adrenal tissue.

By combining the adrenal miRNA-expression data and the bioinformatic target site analysis, those miRNAs which may regulate *CYP11B1* and *CYP11B2* expression in the adrenal gland could be identified. This approach identified 24 miRNAs with putative binding sites on the *CYP11B1* 3'UTR and 16 on *CYP11B2* 3'UTR; 12 miRNAs were common to both. Since these data were compiled, one potential *CYP11B1*-regulating miRNA, miR-768-5p, has been removed from miRBase as the genomic location from which it was transcribed has been re-annotated as

containing a small nucleolar RNA, HBII-239. Evolutionary conservation analysis supports the mapping of HBII-239, therefore this miRNA is excluded from future investigation. One adrenal-miRNA, miR-638, was predicted in three databases, the most of all the miRNAs, and may have represented the best candidate miRNA for further study if qRT-PCR quantification had not shown that it is actually relatively lowly expressed within the adrenal gland.

To identify which miRNA may be likely to regulate these genes it was interesting to note that several of the predicted miRNAs belong to the same miRNA family, e.g. miR-10a and miR-10b, which share the same target site on the 3'UTR. Moreover, other miRNAs which are not part of a miR-family also share common binding regions e.g. miR-23a, miR-23b and miR-181a which all bind to a similar region in the 3'UTR of *CYP11B1*. This may add complexity to the mechanism by which these miRNAs work; a recent study has been the first to demonstrate that more than one miRNA can regulate an mRNA (Wu *et al.*, 2010). Several adrenal miRNAs in the target lists are produced as part of a cluster, and this may represent some sort of adrenal transcriptional specificity. For example, miR-24, miR-23b and miR-27b are all found in the adrenal gland, but only miR-24 and miR-23b have binding sites in the *CYP11B1* and *CYP11B2* genes. Cluster members miR-381 and miR-134 are transcribed together but have putative binding sites on different 3'UTR (*CYP11B1* and *CYP11B2*, respectively).

A literature search found that the previously-reported functions of the miRNAs identified fall into four categories: those involved in cancer, including action on tumour suppressor genes, oncogenes or genes that alter cell cycle regulation; those with association to cardiovascular disease; those with association to neural disease; or those whose function has not yet been established. Numerous miRNAs fall into several of these categories, highlighting that each may act on several mRNA targets (Lewis *et al.*, 2003) and, in some cases, a miRNA may act in a cell-type specific manner (Section 1.7.1).

Finally, in the hope of identifying miRNAs that may be associated with hypertension, the genomic locations of the miRNAs were compared with those of published QTL (quantitative trait loci) associated with hypertension (Cowley, 2006). In total, six miRNAs are encoded in known hypertension QTL and of these, four are located in the QTL of defined causes of hypertension or identified in

non-Caucasian cohorts. Of the remaining two, the sequence of miR-10a lies within an intron of a homeobox gene on chromosome 17 at location identified in the Framingham Heart Study as being associated with hypertension (Levy *et al.*, 2009), while miR-28-5p is found in an intron of the LPP gene, at a region identified in the HERITAGE Family Study (Rice *et al.*, 2002). As previously discussed (Section 1.1.3.2), the identification of QTL for hypertension has proved difficult to replicate and to validate by functional experiments investigating the gene coded within them. However, it may be possible that miRNAs located in QTL may be the functioning regulator of pathways involved in the development of hypertension.

In conclusion, these studies have identified several putative miRNA binding sites in all of the genes involved in corticosteroid synthesis and have defined the miRNA expression profile of a normal human adrenal gland to produce a shortlist of potential miRNAs that may regulate *CYP11B1* and *CYP11B2* mRNA stability. The work presented in this chapter has also highlighted, by sequence conservation analysis and relative 3'UTR length, that the *CYP11B1* and *CYP11B2* genes are good candidates for miRNA regulation. The subset of adrenal-miRNAs which have a putative miRNA target-site on the 3'UTR are currently based on bioinformatic prediction only. Therefore, they require experimental validation which will be presented in Chapter 5 of this thesis.

5 miRNA Target-Site Validation in the 3'UTR of *CYP11B1* and *CYP11B2*.

5.1 Introduction

Putative miRNA binding sites must be experimentally investigated in order to validate bioinformatic analyses and this will be the focus of the current chapter. Validation can be achieved by several methods, most of which involve *in vitro* analysis, that may provide direct or indirect evidence of miRNA-mediated regulation. Two important considerations when designing experiments are that the measurements should be highly specific and accurate to allow for detection of the small changes typical of miRNA-mediated regulation, and that a suitable model be chosen, that best represents the *in vivo* situation.

Methods to validate miRNA target sites typically involve manipulating miRNA-expression levels, then assessing mRNA abundance or protein levels. Several examples of miRNA inhibition are given in Section 1.8 and Figure 1-20. Increasing cellular miRNA levels can be achieved by introducing exogenous miRNA mimics into cells, these can be in the form of short single-stranded RNA molecules, larger molecules which are cleaved intracellularly to form short single-stranded miRNA molecules, or plasmid constructs containing pre-miR or pri-miR sequences that are expressed under the control of a viral or cell-type specific promoter. In this chapter, short single-stranded molecules are used to manipulate miRNA abundance; these include Pre-miR™ molecules, which increase miRNA expression, and Anti-miR™ molecules whose complementarity to specific endogenous miRNAs causes them to be bound and competitively antagonised. The benefits of these molecules are that they do not require endogenous miRNA processing enzymes, such as Dicer, that they are specific to one miRNA (whereas those involving expression of a pre-miR will lead to exogenous expression of miRNA and miRNA* species) and that it is possible to measure their effect on miRNA expression by qRT-PCR analysis.

Two types of miRNA target validation experiment will be used in this chapter: firstly, the 3'UTR reporter plasmid construct used in Chapter 3 will be tested with specific Pre-miR™ or Anti-miR™ molecules and miRNA binding assessed by measuring the relative luciferase levels; secondly, miRNA-action will be assessed by transfecting these molecules into the human adrenocortical cell line, H295R (Section 3.1), and then assessing the levels of *CYP11B1* or *CYP11B2* mRNA by

qRT-PCR. The subsequent effects on steroid production will also be assessed by measuring cortisol and aldosterone secretion in cell media.

An additional parameter that is often investigated in miRNA analysis is the effect on protein synthesis, usually measured by western blot analysis. However, there are currently no antibodies available for detecting the human *CYP11B1* and *CYP11B2* proteins specifically and effectively. Therefore, this could not be performed in this current study, although changes in steroid production can be suggestive of changes in protein abundance.

5.2 Aims

The aims of this study are to investigate the function of adrenal miRNAs predicted to have binding sites on the *CYP11B1* and/or *CYP11B2* 3'UTR with a view to validating the bioinformatic analyses. To achieve this, miRNA targeting of the 3'UTR will be assessed by reporter construct analysis, as determined by measurement of steroids and of *CYP11B1* and *CYP11B2* mRNA following transfection of H295R cells with small molecules capable of modulation miRNA levels (*i.e.* Pre-miR[™] and Anti-miR[™]).

5.3 Materials and Methods

5.3.1 Investigation of miRNA Binding to 3'UTR Reporter Construct

HeLa cells were co-transfected with pEZX reporter constructs (500 ng) and Pre-miR™ or Anti-miR™ molecules (50 nM) in 24-well plates as described in Section 2.9.4. 48 Hours post-transfection, cell lysates were prepared and their firefly and renilla luciferase activities measured using the Dual Reporter Luciferase Assay (DLRA, Sections 2.10). Control transfections and normalisation methods were identical to those described in Section 3.3.3.

5.3.2 Pre-miR™ or Anti-miR™ Transfection of H295R Cells

H295R cells were transfected in 6-well plates as described in Section 2.9. The Pre-miR™ or Anti-miR™ molecules used are listed Table 5-1 and were prepared as detailed in Section 2.9.1. The medium was replaced on the cells after 24 hours and, after 48 hours, this was removed for steroid analysis by LC:MS/MS (Section 2.11). Cell lysates were prepared for total RNA isolation (Section 2.2.1), ready for qRT-PCR analysis as described in Section 2.4 and 2.5. Data were analysed using the $\Delta\Delta C_t$ method (Section 2.5.4), with *GAPDH* or *RNU48* used as a housekeeping gene and results expressed relative to the negative (Pre-miR™ or Anti-miR™) control-transfected cells.

5.3.3 Statistical Analysis

For experiments performed once in technical triplicate statistical analysis was performed using a Student's t-test.

All other experiments were performed at least in triplicate and at least two biologically-independent times. As described above, the miRNA, Pre-miR™ or Anti-miR™ molecules were normalised to a negative control transfected with a scrambled Pre-miR™ or Anti-miR as appropriate. The mean result of the biologically replicated experiments were then taken and statistical analysis was performed using a one-sample t-test, imputing either 100% or a 1-fold change as a reference level to which experimental values were compared.

For mature miRNA measurement following increasing Pre-miR™ or Anti-miR™ concentrations, a one-way ANOVA and Bonferroni's post-hoc tests were used, comparing each concentration to the negative control cells.

For all analyses, confidence intervals of 95% were used and $P < 0.05$ was required for statistical significance. Data are expressed as the mean \pm standard error of the mean (SEM).

Table 5-1. Pre-miR™ and Anti-miR™ molecules.

| miRNA | Pre-miR™ Product Code | Anti-miR™ Product Code |
|---------------------|-----------------------|------------------------|
| Negative Control #1 | AM17110 | AM17010 |
| Let-7c | | 4392431 |
| miR-1 | AM17150 | |
| miR-10a | PM10787 | AM10787 |
| miR-10b | PM11108 | AM11108 |
| miR-125a-5p | PM12561 | AM12561 |
| miR-125b | PM10148 | AM10148 |
| miR-134 | PM10341 | AM10341 |
| mir-140-3p | PM12503 | AM12503 |
| miR-143 | PM10883 | AM10883 |
| miR-185 | PM12486 | AM12486 |
| miR-22 | PM10203 | AM10203 |
| miR-23b | PM10711 | AM10711 |
| miR-24 | PM10737 | AM10737 |
| miR-34a | PM11030 | AM11030 |
| miR-382 | PM10924 | AM10924 |
| miR-423-5p | PM11164 | AM11164 |
| miR-495 | PM11526 | AM11526 |
| miR-638 | PM11594 | AM11594 |
| miR-940 | PM12798 | AM12798 |

Supplier: Applied Biosystems (Warrington, U.K.)

5.4 Results

5.4.1 Assessing Potential Repression of the *pEZX* Reporter Construct

To investigate the suitability of the 3'UTR reporter construct as an experimental tool, a siRNA molecule with perfect complementarity was designed to target each 3'UTR (Table 5-2). Each was co-transfected into HeLa cells with the corresponding reporter construct. The ratio of firefly luciferase to renilla luciferase was calculated and normalised to a transfected control siRNA. The *CYP11B1* 3'UTR siRNA significantly decreased the normalised ratio from the 100% control level to $16.13\% \pm 0.67$ (Figure 5-1A). Similarly, the *CYP11B2* 3'UTR siRNA significantly decreased the ratio to $52.34\% \pm 4.51$ relative to the control level of 100% (Figure 5-1B). These results offered proof of concept that miRNAs targeting the 3'UTR of either mRNA will result in significantly different luciferase activity that can be measured with a good degree of sensitivity.

Table 5-2. siRNA Molecules Targeted to the *CYP11B1* and *CYP11B2* 3'UTR.

| siRNA Name | Sequence |
|---------------|---------------------|
| CYP11B1 siRNA | UCUACAUCCUUCUCAUAUA |
| CYP11B2 siRNA | AUACUUAGGUAAUCAUUC |

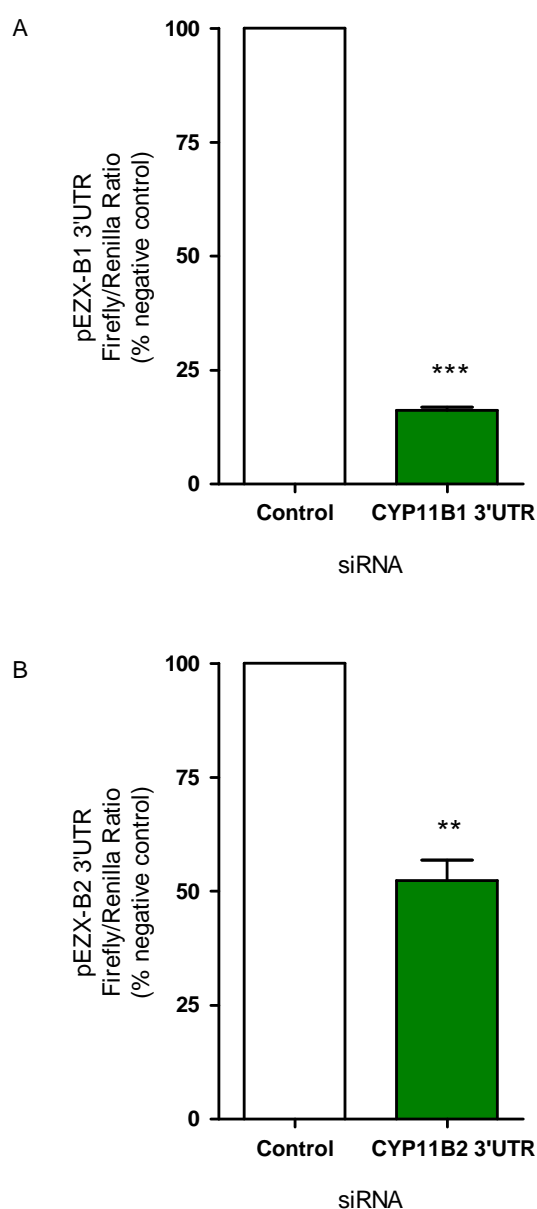


Figure 5-1. siRNA Verification of the pEZX-B1 and pEZX-B2 Reporter Plasmids.

HeLa cells were co-transfected with (A) pEZX-B1 construct and *CYP11B1* 3'-UTR siRNA or (B) pEZX-B2 construct and *CYP11B2* 3'UTR siRNA. Firefly and renilla luciferase were measured 48 hours post-transfection using the Dual Luciferase Reporter Assay Kit. To normalise for transfection efficiency the ratio of firefly to renilla was measured and expressed as a percentage of the negative control siRNA value. Results represent the mean of three independent biological experiments, performed in quadruplicate; error bars represent standard error of the mean (SEM). ** $p < 0.01$, *** $p < 0.001$ compared to control.

5.4.2 miRNA Targeting to the 3'UTR of *CYP11B1* mRNA.

miRNAs with putative binding sites in the 3'UTR of both *CYP11B1* and *CYP11B2* mRNA (Figure 4-8) were tested in turn, using the pEZX-reporter constructs to establish if the miRNA bind to the 3'UTR and cause repression. Specific single-stranded Pre-miR™ or Anti-miR™ were co-transfected into HeLa cells alongside the reporter construct to either overexpress or competitively inhibit a particular miRNA species.

Manipulating the levels of: miR-10a, miR-10b, miR-140-3p, miR-143, miR-22 and miR-34a did not change the relative levels of luciferase activity (Figure 5-2A-D: F and Figure 5-3B). Therefore, experimental data failed to validate the predicted bioinformatic target sites for these miRNAs on the *CYP11B1* 3'UTR.

However, several other miRNAs did cause changes in the relative luciferase levels. Firstly, increasing miR-185, reduced luciferase activity to $79.50 \pm 4.35\%$ ($p = 0.018$) although inhibiting the same miRNA with an Anti-miR™ did not increase the level of luciferase activity (Figure 5-2E). miR-24 (Figure 5-3A) and miR-638 (Figure 5-3E) a similarly consistent pattern of results with neither producing a change in luciferase activity when overexpressed despite seeing an increase in Anti-miR™ transfected cells: miR-24 increased luciferase activity to $137.30 \pm 5.87\%$ ($p = 0.023$) and miR-638 increased it to $144.8 \pm 4.65\%$ ($p = 0.066$). The putative miR-382 binding site on the *CYP11B1* 3'UTR was confirmed by changes in luciferase activity characteristic of canonical miRNA action *i.e.* Pre-miR™ decreased luciferase activity to $58.51 \pm 2.63\%$ ($p = 0.004$) and Anti-miR™ transfected cells increased it to $121.90 \pm 3.22\%$ ($p = 0.021$) of control cells (Figure 5-3C). A similar pattern was observed for miR-940 (Figure 5-3F): Pre-miR™ caused a large decrease in luciferase activity to $34.14 \pm 2.25\%$ ($p = 0.0012$). However, the Anti-miR™ only marginally increased it to $121.40 \pm 8.98\%$ and did not reach statistical significance. Manipulation of miR-423-5p caused interesting results: Anti-miR™ cells did not cause a change in luciferase activity but Pre-miR™ increased it to $186.50 \pm 8.015\%$ ($p = 0.0085$). While this result does not confirm the existence of a miRNA target site it may suggest some other type of regulation (Figure 5-3D).

A summary of these results is shown in Table 5-3.

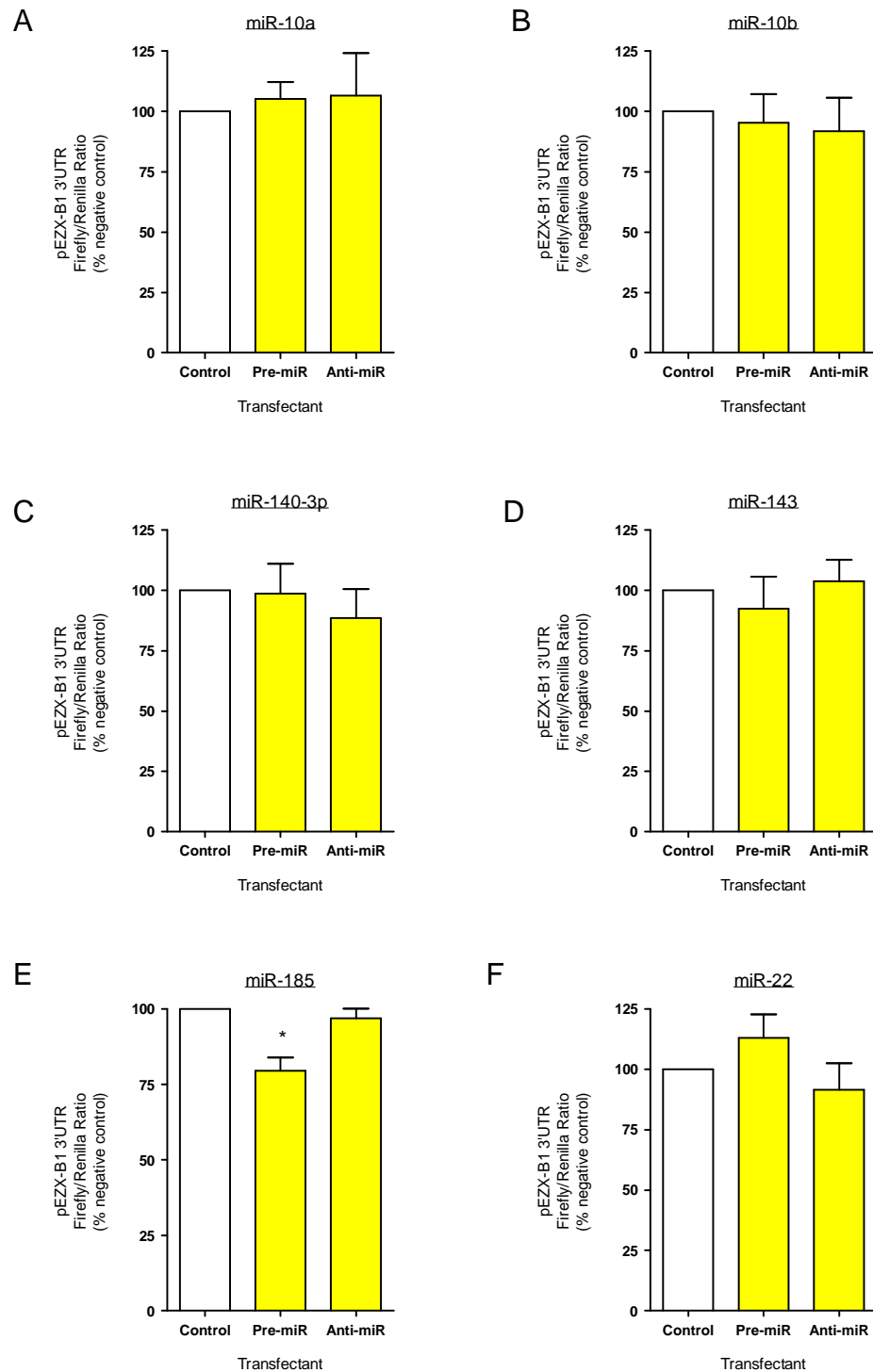


Figure 5-2. Effect of miRNAs on the Luciferase Activity of the pEZXB1 Reporter Construct.

HeLa cells were co-transfected with the pEZXB1 construct and Pre-miR™ or Anti-miR™ molecules for the indicated miRNAs. Firefly and renilla luciferase activity were measured 48 hours post-transfection using the Dual Luciferase Reporter Assay System. To normalise for transfection efficiency, the ratio of firefly to renilla luminescence was calculated and expressed as a percentage of the negative, scrambled control. Results represent the mean of at least three independent biological experiments, performed in quadruplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$.

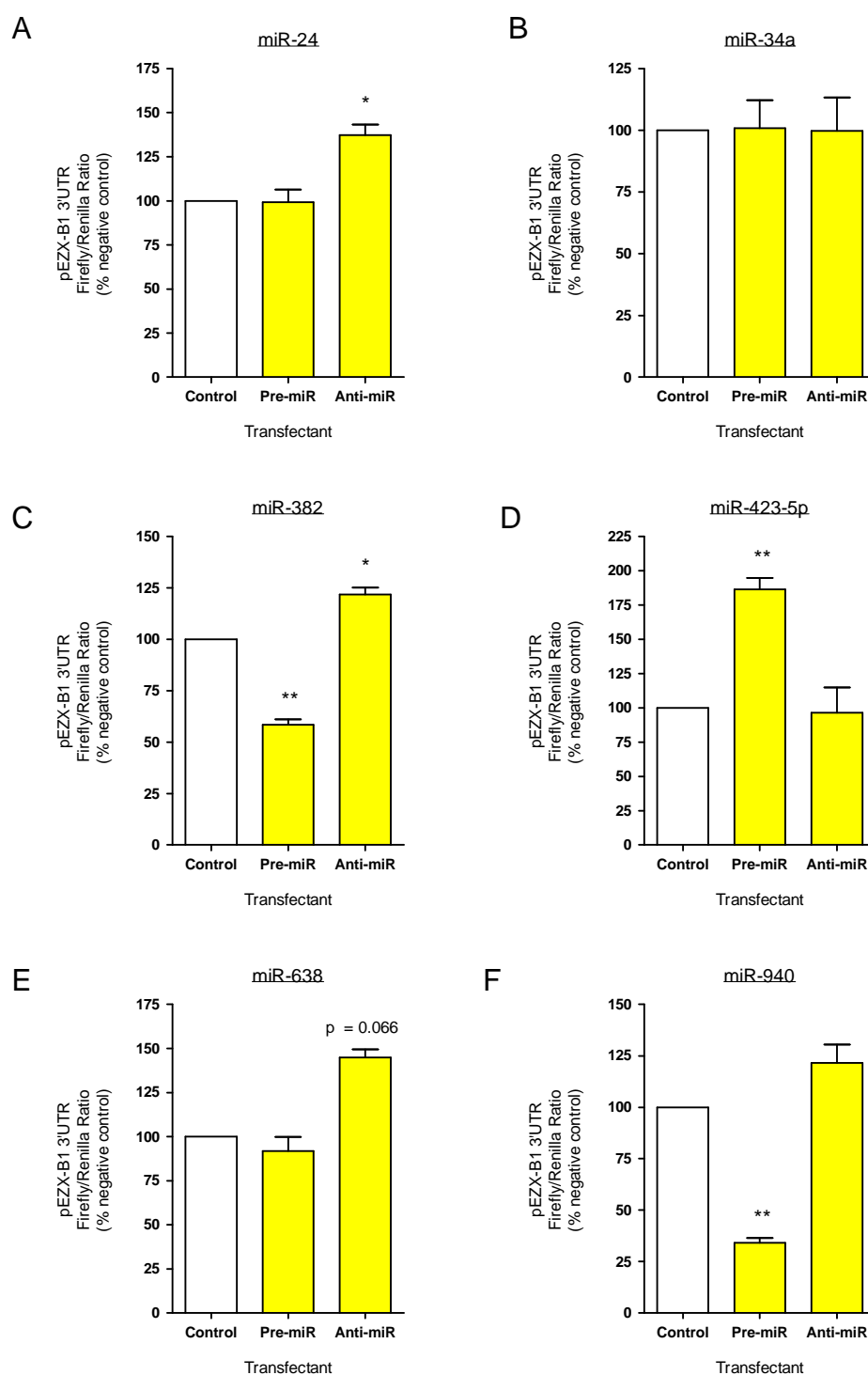


Figure 5-3. Effect of miRNAs on the Luciferase Activity of the pEZXB1 Reporter Construct (2).

HeLa cells were co-transfected with the pEZXB1 construct and Pre-miR™ or Anti-miR™ molecules for the indicated miRNAs. Firefly and renilla luciferase activity were measured 48 hours post-transfection using the Dual Luciferase Reporter Assay System. To normalise for transfection efficiency, the ratio of firefly to renilla luminescence was calculated and expressed as a percentage of the negative, scrambled control. Results represent the mean of at least three independent biological experiments, performed in quadruplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$, ** < 0.01 or p-value as stated.

5.4.3 miRNA Targeting to the 3'UTR of *CYP11B2* mRNA.

Bioinformatic target site predictions on the *CYP11B2* mRNA were tested using the pEZXB2 reporter construct. These experiments failed to confirm target-site predictions for miR-10a (Figure 5-4A), miR-10b (Figure 5-4B), miR-140-3p (Figure 5-4C), miR-22 (Figure 5-4F), miR-382 (Figure 5-5C) miR-423-5p (Figure 5-5D) or miR-638 (Figure 5-5E), none of which caused a significant change in luciferase activity when either overexpressed or inhibited in HeLa cells. However, in some circumstances (e.g. miR-22, miR-384 and miR-423-5p), large changes in luciferase activity were observed, but which failed to reach statistical significance, probably due to large standard deviations in the replicate results (Figure 5-4 and Figure 5-5).

In contrast, the results for miR-143 indicate that it does bind to the 3'UTR of *CYP11B2* (Figure 5-4D); increasing miR-143 significantly reduced luciferase activity to $79.03 \pm 4.86\%$ ($p = 0.499$), while inhibiting miR-143 increased luciferase activity, $128.50 \pm 9.57\%$ although this failed to reach statistical significance ($p = 0.093$). A similar pattern of results was observed for miR-185, with the Pre-miRTM significantly reducing luciferase activity ($64.06 \pm 4.12\%$; $p = 0.003$), and the Anti-miRTM increasing luciferase activity ($145.10 \pm 10.91\%$) but without reaching statistical significance ($p = 0.054$; Figure 5-4E).

Increasing miR-24 did not change the luciferase activity of the pEZXB2 reporter construct in HeLa cells but inhibiting miR-24 with the Anti-miRTM molecule caused it to increase significantly to $162.80 \pm 11.20\%$ ($p = 0.030$, Figure 5-5A). Results also show that miR-34a overexpression reduce luciferase activity to $68.52 \pm 7.20\%$ ($p = 0.485$, Figure 5-5B) but there was no change in Anti-miRTM-transfected cells. A similar pattern was observed for miR-940 (Figure 5-5F) where Pre-miRTM reduced luciferase activity by a large margin ($27.23 \pm 5.23\%$; $p = 0.0081$) but Anti-miRTM did not achieve significance (123.00 ± 8.00 ; $p = 0.1028$).

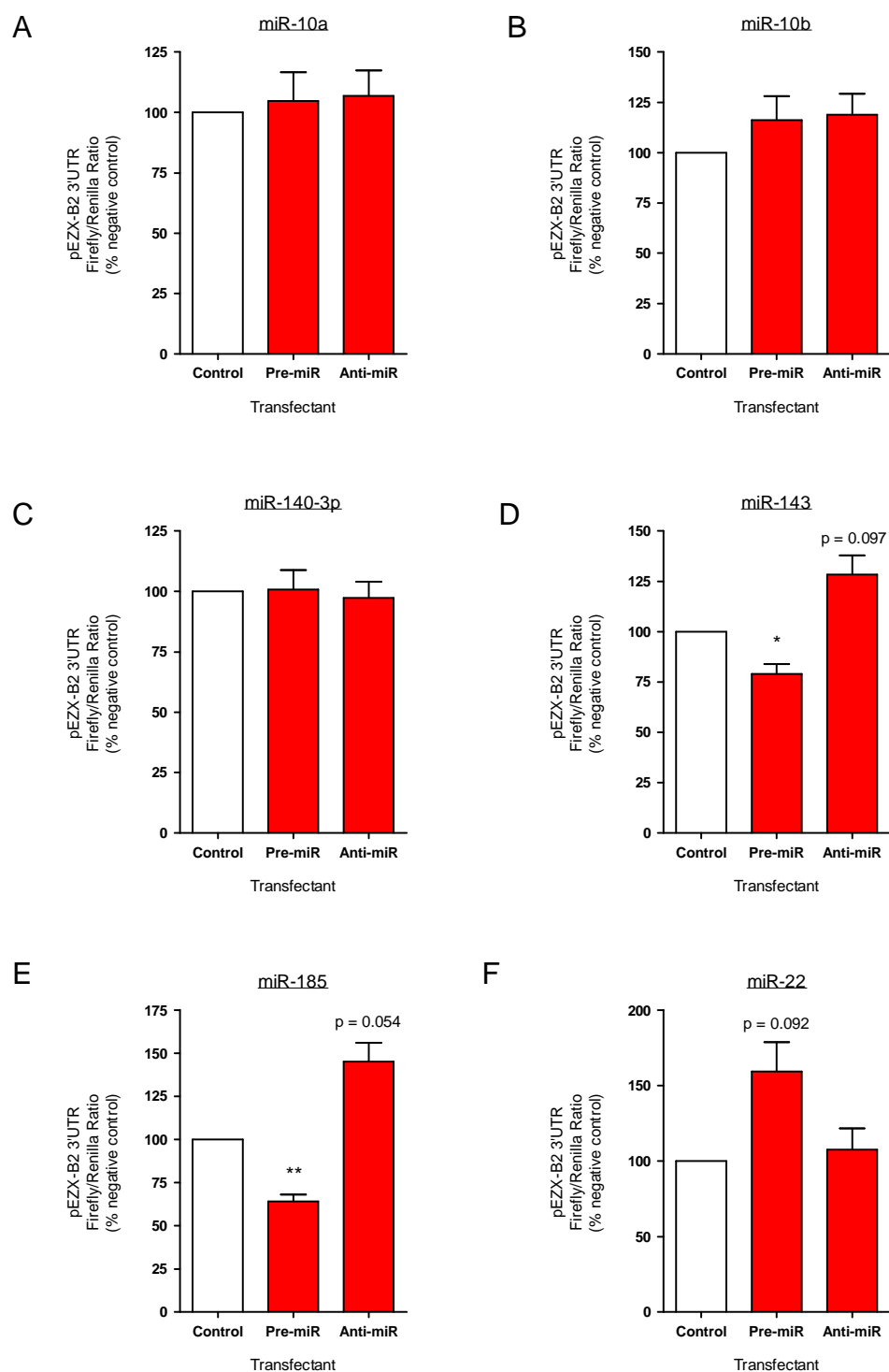


Figure 5-4. Effect of miRNAs on the Luciferase Activity of the pEZXB2 Reporter Construct.

HeLa cells were co-transfected with the pEZXB1 construct and Pre-miR™ or Anti-miR™ molecules for the indicated miRNAs. Firefly and renilla luciferase activity were measured 48 hours post-transfection using the Dual Luciferase Reporter Assay System. To normalise for transfection efficiency, the ratio of firefly to renilla luminescence was calculated and expressed as a percentage of the negative, scrambled control. Results represent the mean of at least three independent biological experiments, performed in quadruplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$, ** < 0.01 or p-value as stated.

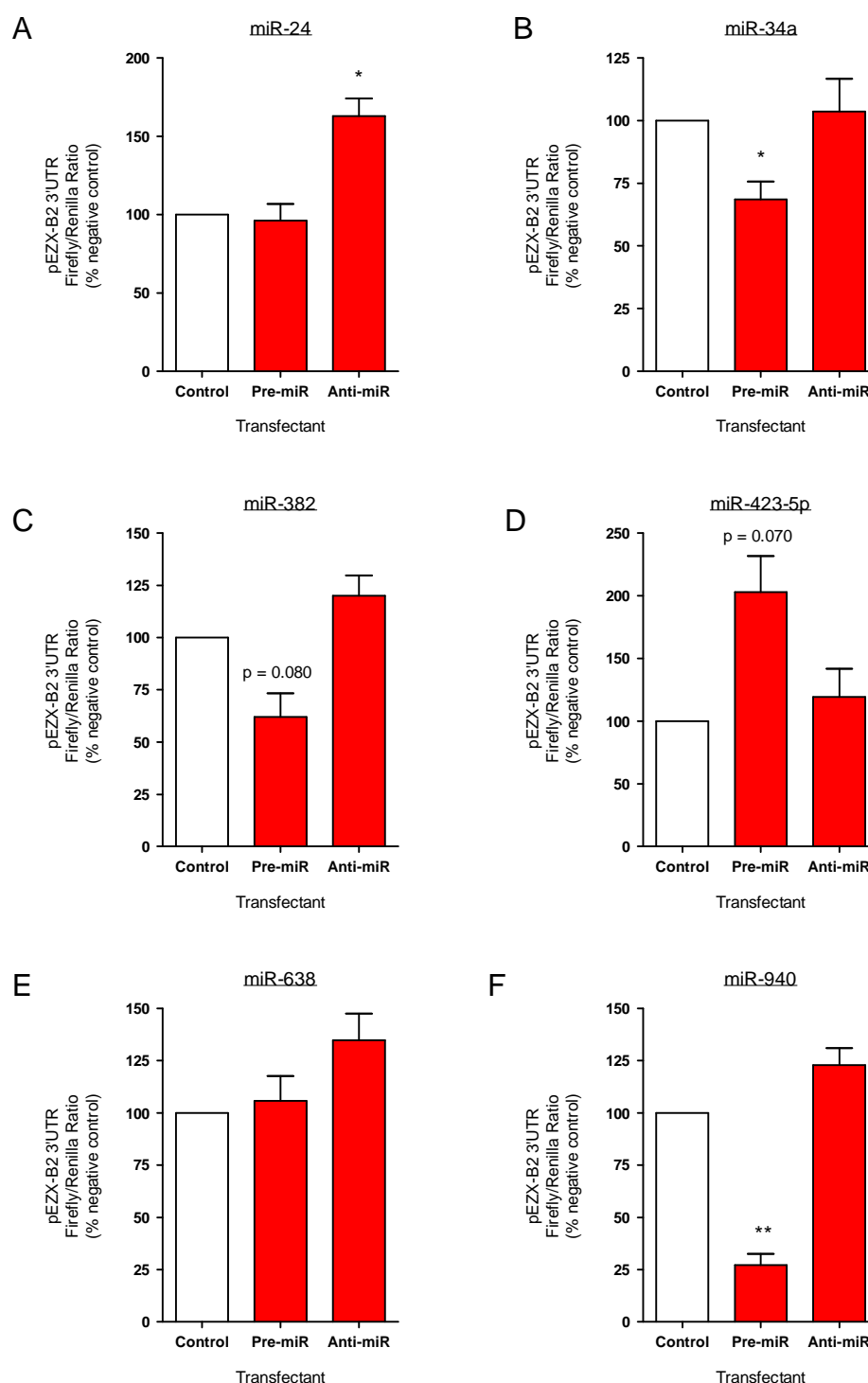


Figure 5-5. Effect of miRNAs on the Luciferase Activity of the pEZXB2 Reporter Construct [2].

HeLa cells were co-transfected with the pEZXB1 construct and Pre-miR™ or Anti-miR™ molecules for the indicated miRNAs. Firefly and renilla luciferase activity were measured 48 hours post-transfection using the Dual Luciferase Reporter Assay System. To normalise for transfection efficiency, the ratio of firefly to renilla luminescence was calculated and expressed as a percentage of the negative, scrambled control. Results represent the mean of at least three independent biological experiments, performed in quadruplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$, ** < 0.01 or p-value as stated.

5.4.4 miRNAs with Unique Target Sites on the *CYP11B2* 3'UTR.

In addition to testing those miRNAs which had putative miRNA binding sites in *CYP11B1* and *CYP11B2*, the four miRNAs which were predicted only to bind the *CYP11B2* 3'UTR (Figure 4-8) were also tested using the same system. The results of these experiments are shown in Figure 5-6.

Manipulating the levels of miR-134 or miR-495 did not significantly alter the luciferase activity of the pEZXB2 reporter construct (Figure 5-6C-D). However, the existence of a miRNA binding site for miR-125a-5p and miR-125b on the *CYP11B2* 3'UTR was confirmed by these experiments; increasing the levels of miR-125a-5p in Pre-miR™ transfected cells reduced luciferase activity to a non-significant level of $63.78 \pm 8.09\%$ but decreasing miR-125a-5p in Anti-miR™ transfected cells significantly increased luciferase activity to $143.50 \pm 9.02\%$ ($p = 0.040$, Figure 5-6A). miR-125b Pre-miR™ transfected cells had significantly decreased luciferase activity ($75.90 \pm 1.28\%$; $p = 0.033$) and conversely, the Anti-miR™ transfected cells produced a significant increase in luciferase activity (148.70 ± 7.61 ; $p = 0.023$), in line with canonical miRNA action (Figure 5-6B).

A summary of the results for all miRNAs targeting the *CYP11B2* 3'UTR are shown in Table 5-4 (page 227).

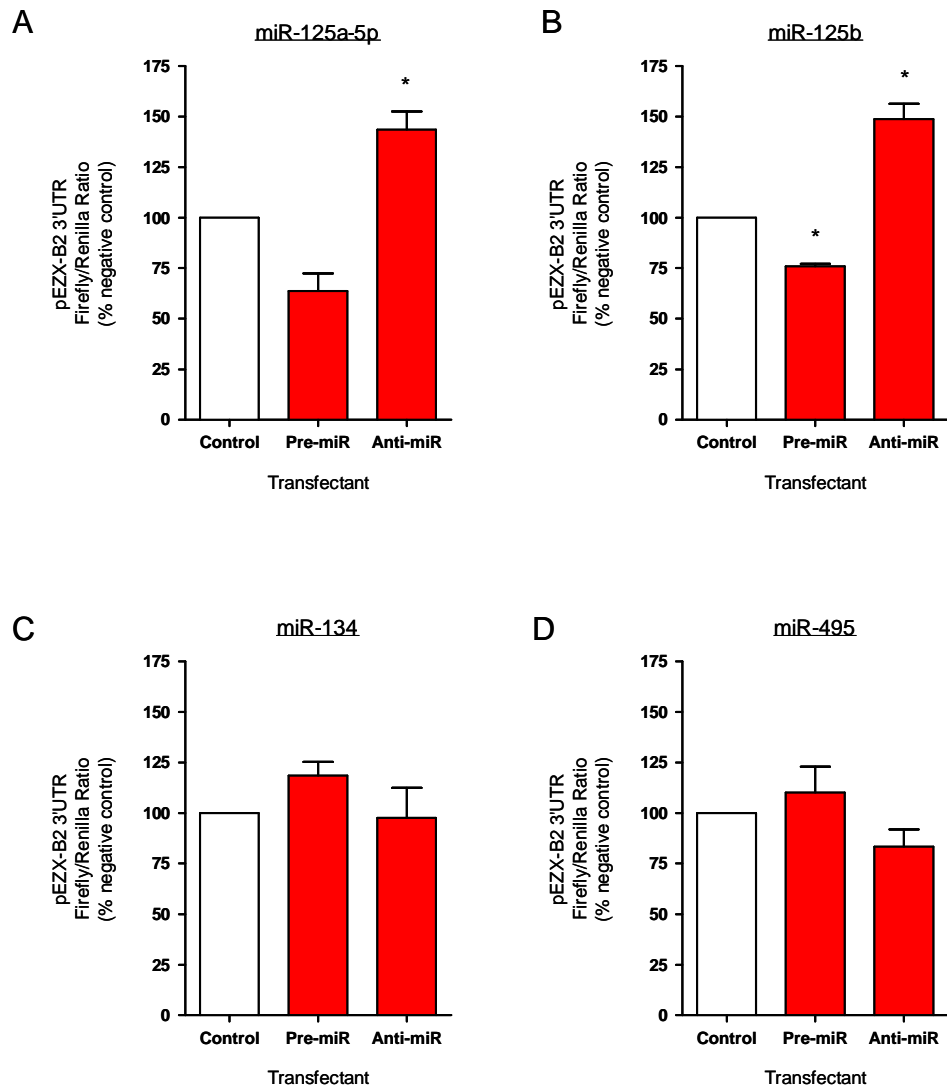


Figure 5-6. Effect of *CYP11B2*-Specific Targeting miRNAs on the Luciferase Activity of the pEZXB2 Reporter Construct.

HeLa cells were co-transfected with the pEZXB1 construct and Pre-miR™ or Anti-miR™ molecules for the indicated miRNAs. Firefly and renilla luciferase activity were measured 48 hours post-transfection using the Dual Luciferase Reporter Assay Kit. To normalise for transfection efficiency, the ratio of firefly to renilla luminescence was calculated and expressed as a percentage of the negative, scrambled control. Results represent the mean of at least three independent biological experiments, performed in quadruplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$.

5.4.5 Assessment of H295R Transfection with Pre-miR™ and Anti-miR™ Molecules.

To investigate the action of miRNA on the full-length *CYP11B1* or *CYP11B2* mRNAs, the Pre-miR™ and Anti-miR™ molecules were transfected into the H295R adrenocortical cell line and their effects assessed by measuring the relative abundance of *CYP11B1* or *CYP11B2* mRNA and the levels of steroids secreted into the cell media.

First, it was necessary to assess the viability of using these small molecules and the suitability of the transfection conditions for H295R cells. A positive control experiment was performed and the levels of mature miRNA measured following transfection with three different concentrations of miR-24 Pre-miR™ or Anti-miR™.

Figure 5-7 shows the levels of mature miR-24 measured by qRT-PCR following transfection of 1, 10 or 50 nM of the miR-24 Pre-miR™ or Anti-miR™. A concentration-dependent increase in mature miR-24 was observed (Figure 5-7A). 1 nM increased the levels 50.24 ± 2.73 -fold compared to control transfected cells but this did not reach statistical significance; 10 nM and 50 nM concentrations increased the level of mature miR-24 by 499.79 ± 67.46 ($p < 0.05$) and 1914.04 ± 225.22 ($p < 0.001$) fold, respectively. Similarly, the levels of mature miR-24 decreased in a concentration-dependent manner (1 nM: 0.705 ± 0.009 ; 10 nM: 0.027 ± 0.003 ; 50 nM: 0.023 ± 0.007 , with results statistically different relative to the scrambled, negative control (Figure 5-7B).

The extent to which different miRNAs were overexpressed relative to their basal level was compared by transfecting H295R cells with Pre-miR™ molecules for five different miRNAs and then measuring the levels of those miRNAs. Figure 5-8 shows the results for miR-10b, miR-125a-5p, miR-21, miR-24 and miR-143, all transfected at a final concentration of 50 nM. All miRNAs tested saw an increase, and there was a large range of fold-changes in the miRNA levels; the extent of this change may be influenced strongly by the basal level of the miRNA in H295R cells.

These results suggests that transfection of H295R cells with small molecules was successful and that all Pre-miR[™] molecules are suitable for increasing their specific miRNAs and while Anti-miR[™] molecules are capable of decreasing mature miRNA levels in a specific manner. The magnitude of change caused by Pre-miR[™] transfection does vary depending on the individual miRNA species and the basal endogenous levels however, in general, there is a significant and large change with a 50 nM concentration of Pre-miR[™] or Anti-miR[™].

To assess the ability of Pre-miR[™] or Anti-miR[™] to modulate mRNA targets, positive control experiments were set up to measure the effect of miR-1 Pre-miR[™] transfection on its validated target, *PTK9* (Lim *et al.*, 2005), and of let-7c Anti-miR[™] on its target, *HMGA2* (Lee and Dutta, 2007). Increasing the levels of miR-1 successfully decreased the level of *PTK9* mRNA abundance to 0.56 ± 0.05 fold ($p = 0.004$, Figure 5-9A), while decreasing the levels of let-7c increased *HMGA2* mRNA by 1.47 ± 0.06 fold ($p = 0.014$, Figure 5-9B). These results matched the expected results, as set out by the manufacturer, and confirmed that the transfection protocol is appropriate for assessing miRNA function in the H295R cell. It was therefore adopted for the subsequent experiments described in this chapter.

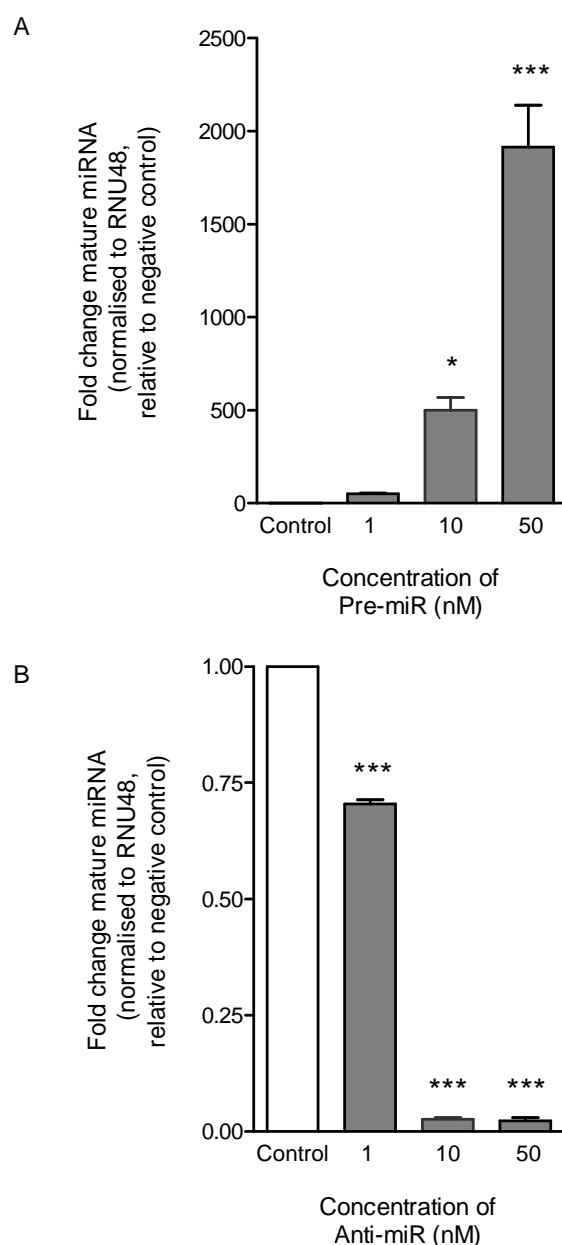


Figure 5-7. Mature miR-24 Levels Following Transfection with Various Concentrations of miR-24 Pre-miR™ or Anti-miR™.

H295R cells were transfected with (A) miR-24 Pre-miR™ or (B) miR-24 Anti-miR™ and equivalent scrambled negative controls at 1, 10 and 50 nM. Mature miR-24 levels were measured 48 hours post-transfection by qRT-PCR. Cycle threshold values were normalised to RNU48 mRNA and expressed relative to the control, scrambled Pre-miR™ or Anti-miR Pre-miR™, cells. Results represent the mean of one independent biological experiments performed in triplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$, *** $p < 0.001$ compared to control.

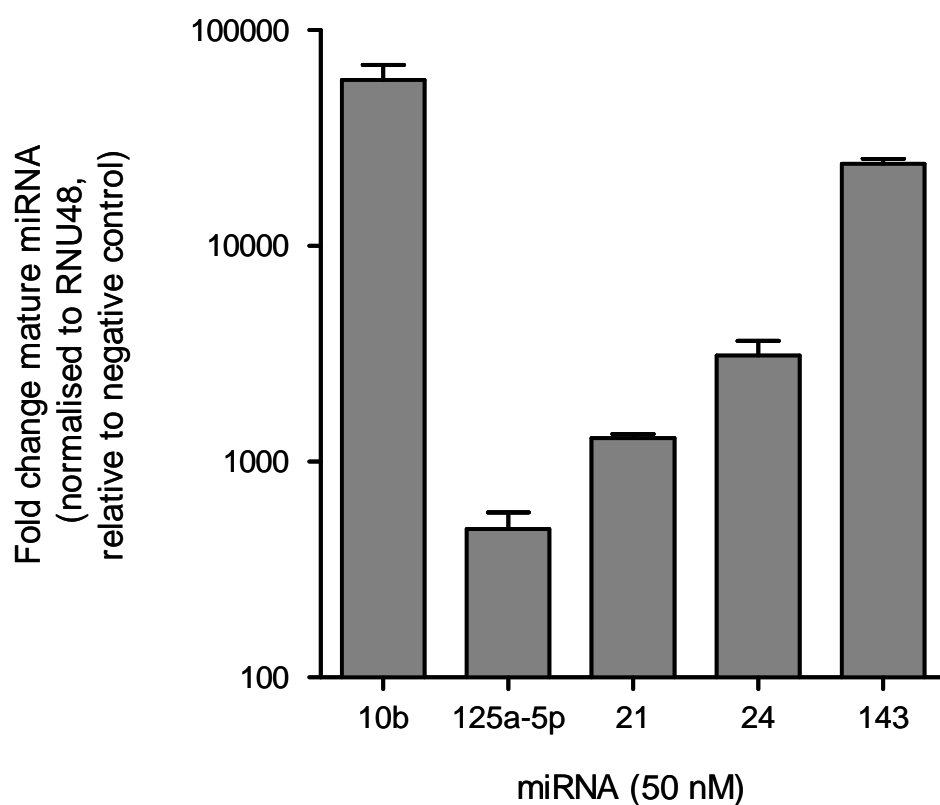


Figure 5-8. Mature miRNAs Levels Post-Transfection in H295R Cells.

H295R cells were transfected with miR-10b, miR-125-5p, miR-21, miR-24 and miR-143 Pre-miR™ and a scrambled negative control (final concentration 50 nM). Mature miRNA levels were measured 48 hours post-transfection by qRT-PCR. Cycle threshold values were normalised to RNU48 mRNA and expressed relative to the negative control cells. Results represent the mean of one independent biological experiments performed in triplicate; error bars represent standard error of the mean (SEM).

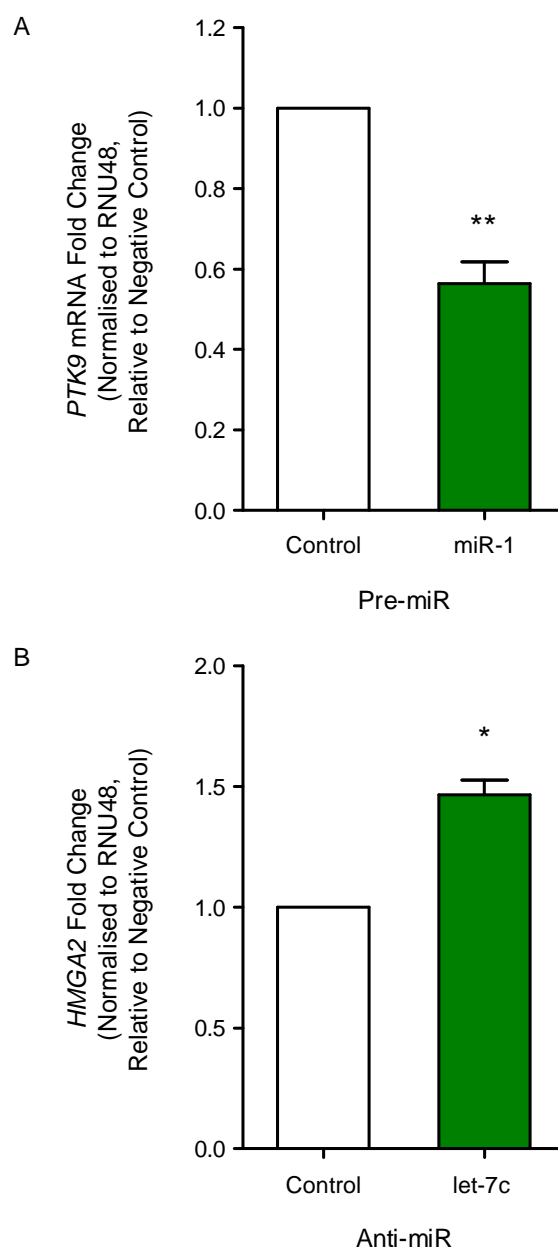


Figure 5-9. Verification of Pre-miR™ and Anti-miR™ Action Using Positive Control Target Genes.

H295R cells were transfected with (A) miR-1 Pre-miR™, (B) let-7c Anti-miR Pre-miR™ or with a scrambled negative control (all final concentration 50 nM). (A) *PTK9* mRNA and (B) *HMGA2* mRNA levels were measured 48 hours post-transfection by qRT-PCR. Cycle threshold values were normalised to RNU48 mRNA and expressed relative to the control cells. Results represent the mean of three independent biological experiments, performed in triplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$ and ** $p < 0.01$ compared to control.

5.4.6 Assessment of mRNA effect on *CYP11B1* and *CYP11B2* mRNA Abundance and Steroid Production.

Based on the results of the reporter construct studies described earlier in this chapter several miRNAs were chosen for further investigation in the H295R cell line, which is the best available *in vitro* model for adrenocortical tissue. The miRNA selected were miR-140-3p, miR-143, miR-185, miR-382, miR-24, miR-628 and miR-940.

miR-10b was also investigated using its specific Pre-miR™ molecule. As it did not change luciferase activity when tested on the pEZX-B1 or pEZX-B2 reporter construct, its use in H295R cells acted as an internal negative control to verify the reporter construct experiments. As expected, increasing miR-10b levels through in Pre-miR™-transfected cells did not significantly change the levels of *CYP11B2* mRNA abundance (1.02 ± 0.12 fold, $p = 0.88$, Figure 5-10A) or *CYP11B1* mRNA abundance (1.10 ± 0.51 , $p = 0.58$, Figure 5-10C); Neither aldosterone nor cortisol levels were significantly altered (Figure 5-10B and D). Therefore, both pEZX reporter constructs and H295R miRNA transfection experiments show that miR-10b does not regulate the *CYP11B1* or *CYP11B2* genes and provide evidence that these two experiment protocols provide consistent data for assessing miRNA action.

Due to some constraints (e.g. difficult cell line), many of these experiments were only performed once in triplicate; precise details can be found in the figure legends and further replication would be required to provide stronger evidence of miRNA action.

miR-140-3p Pre-miR™ transfected H295R cells had significantly reduced levels of *CYP11B2* mRNA (0.70 ± 0.03 fold of control transfected cells; $p = 0.009$) while Anti-miR™ transfected cells increased to *CYP11B2* mRNA 1.18 ± 0.05 fold ($p = 0.095$, Figure 5-11A). These mRNA changes were not supported by aldosterone levels, which were unchanged for both Pre-miR™ and Anti-miR™ cells (Figure 5-11B). The pattern of results for *CYP11B1* mRNA and cortisol production were inconsistent and, as no single result reached statistical significance, no definitive conclusion can be drawn (Figure 5-11C and D).

miR-143-transfected cells did not significantly alter *CYP11B2* or *CYP11B1* mRNA abundance, although there was a trend towards decreased *CYP11B2* levels in Pre-miR™ transfected cells (0.85 ± 0.03 fold; $p = 0.055$, Figure 5-12A). While cortisol levels reflected the lack of change in *CYP11B1* mRNA (Figure 5-12C and D), the aldosterone levels showed a trend towards increased production in Pre-miR™ transfected cells (1.61 ± 0.15 , $p = 0.077$). This conflicts with the mRNA and reporter construct results, and with canonical miRNA action. Aldosterone production in Anti-miR™ transfected cells was not significantly different from control (Figure 5-12B).

H295R cells transfected with miR-185 Pre-miR™ or Anti-miR™ did not exhibit a significant change in either *CYP11B2* mRNA abundance or aldosterone production (Figure 5-13A and B). However, both the Pre-miR™ and Anti-miR™ transfected cells showed a trend towards decreased *CYP11B1* mRNA abundance (0.77 ± 0.04 fold; $p = 0.055$ and 0.58 ± 0.16 fold; $p = 0.070$, Figure 5-13C), with great variability in Anti-miR™ results. Cortisol production levels were unchanged with either molecule (Figure 5-13D). Further replicates would be required to definitively determine miR-185's effects.

The results of miR-24 Pre-miR™ or Anti-miR™ transfection into H295R cells support a role for miR-24 in the regulation of both *CYP11B1* and *CYP11B2* mRNA levels; the experiments involving miRNA were carried out three independent biological times each time in triplicate and the results are shown in Figure 5-14. Pre-miR™ transfection reduced *CYP11B2* levels to 0.58 ± 0.07 fold of control levels, which was significant ($p = 0.026$); moreover, this change was corroborated by a significant decrease in aldosterone production (0.78 ± 0.02 ; $p = 0.044$). The opposite effect was observed with Anti-miR transfection: a significant increase in *CYP11B2* mRNA abundance (1.32 ± 0.06 fold; $p = 0.035$) and in aldosterone production (1.22 ± 0.03 fold; $p = 0.014$, Figure 5-14A and B). Similar observations were made for *CYP11B1* mRNA abundance and cortisol production: Pre-miR™ transfected cells had significantly reduced *CYP11B1* mRNA levels (0.638 ± 0.06 fold; $p = 0.037$) and cortisol production (0.71 ± 0.05 fold; $p = 0.033$). The Anti-miR™ transfected cells had increased *CYP11B1* mRNA levels (1.38 ± 0.05 fold; $p = 0.02$) but no change in cortisol (0.93 ± 0.03 fold; $p = 0.144$, Figure 5-14C and D).

The results for miR-382 were ambiguous; overall, the only significant result was a small increase in *CYP11B2* mRNA following Anti-miR™ transfection (1.19 ± 0.02 fold; Figure 5-15A). However, Pre-miR™ also increased *CYP11B2* abundance by a similar magnitude, although this was not statistically significant, due to a large variability in the replicates. The aldosterone results were also quite variable and, statistically, neither Pre-miR™ nor Anti-miR™ significantly changed aldosterone production in H295R cells (Figure 5-15B). There was also no significant change in *CYP11B1* mRNA or cortisol production (Figure 5-15C and D).

There was a small but significant increase in *CYP11B2* mRNA abundance following in miR-638 Pre-miR™ transfection of H295R cells (1.16 ± 0.03 fold; $p = 0.047$) together with a large increase in aldosterone production (1.63 ± 0.21 fold) although this was not statistically significant (Figure 5-16A and B). The Anti-miR™ transfected cells saw no significant changes in *CYP11B2* mRNA or aldosterone (Figure 5-16A and B). *CYP11B1* and cortisol production were also unaffected by miR-638 (Figure 5-16C and D).

Results following transfection of H295R cells with miR-940 Pre-miR™ and Anti-miR™ molecules do support a regulatory effect on *CYP11B2* mRNA; levels changed to 0.79 ± 0.01 fold ($p = 0.008$) and 1.46 ± 0.05 fold ($p = 0.009$), respectively (Figure 5-17A and B). There was large variability in the aldosterone levels and no significant difference was seen in the Pre-miR™ transfected cells nor in Anti-miR™ transfected cells (1.47 ± 0.12 fold; $p = 0.090$, Figure 5-17A and B). The existence of a miR-940 binding site in *CYP11B1* 3'UTR was not confirmed by these transfection experiments, as there was no significant change in mRNA abundance or cortisol production following Pre-miR™ or Anti-miR™ transfection (Figure 5-17C and D).

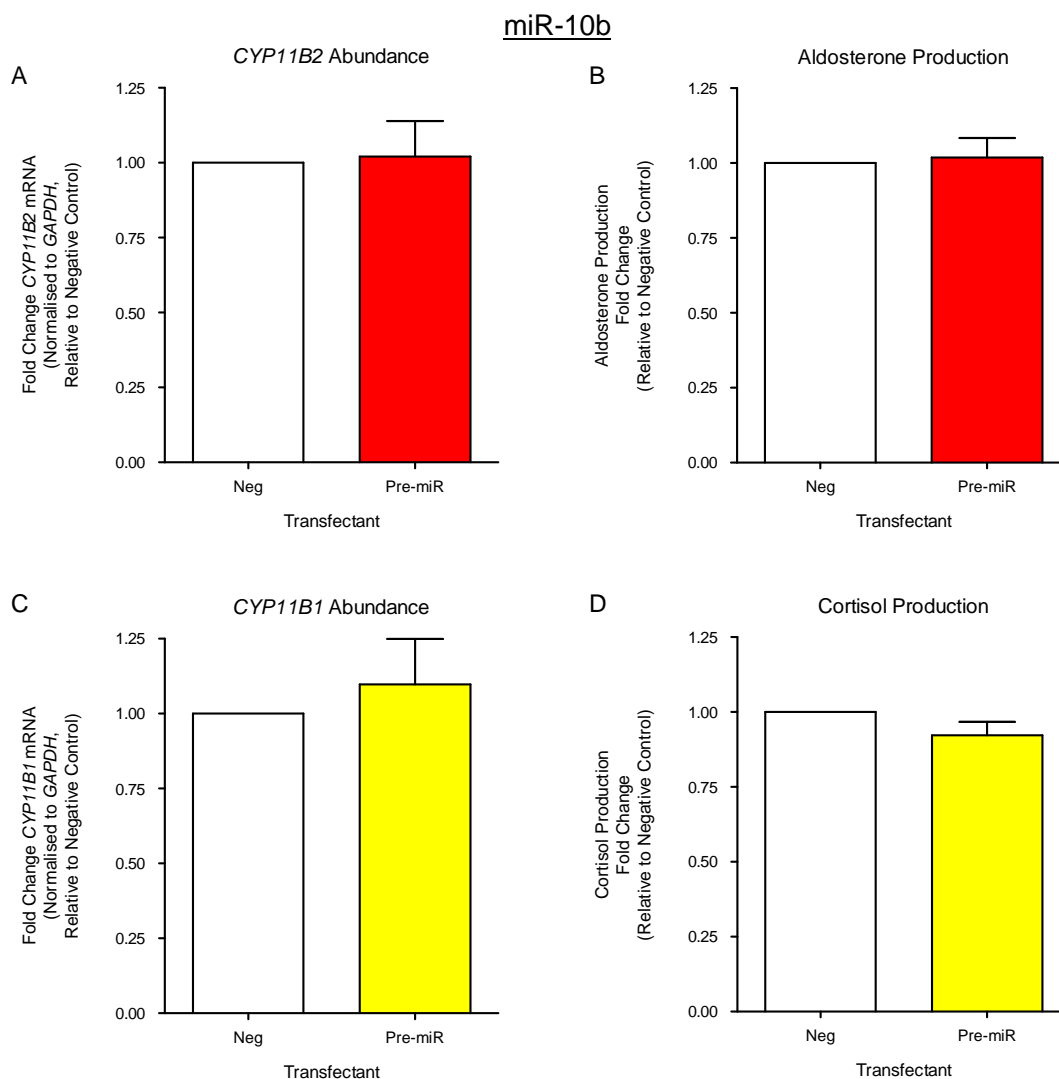


Figure 5-10. Assessment of miR-10b Effects on *CYP11B1* and *CYP11B2* mRNA Levels and on Steroid Production.

H295R cells were transfected with miR-10b Pre-miR™ or with a scrambled negative control miRNA (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* (A) and *CYP11B1* (C) mRNA were measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The levels of aldosterone (B) and cortisol (D) secretion were measured by LC:MS/MS. The results represent the mean of three independent biological experiments performed in triplicate; error bars represent standard error of the mean (SEM).

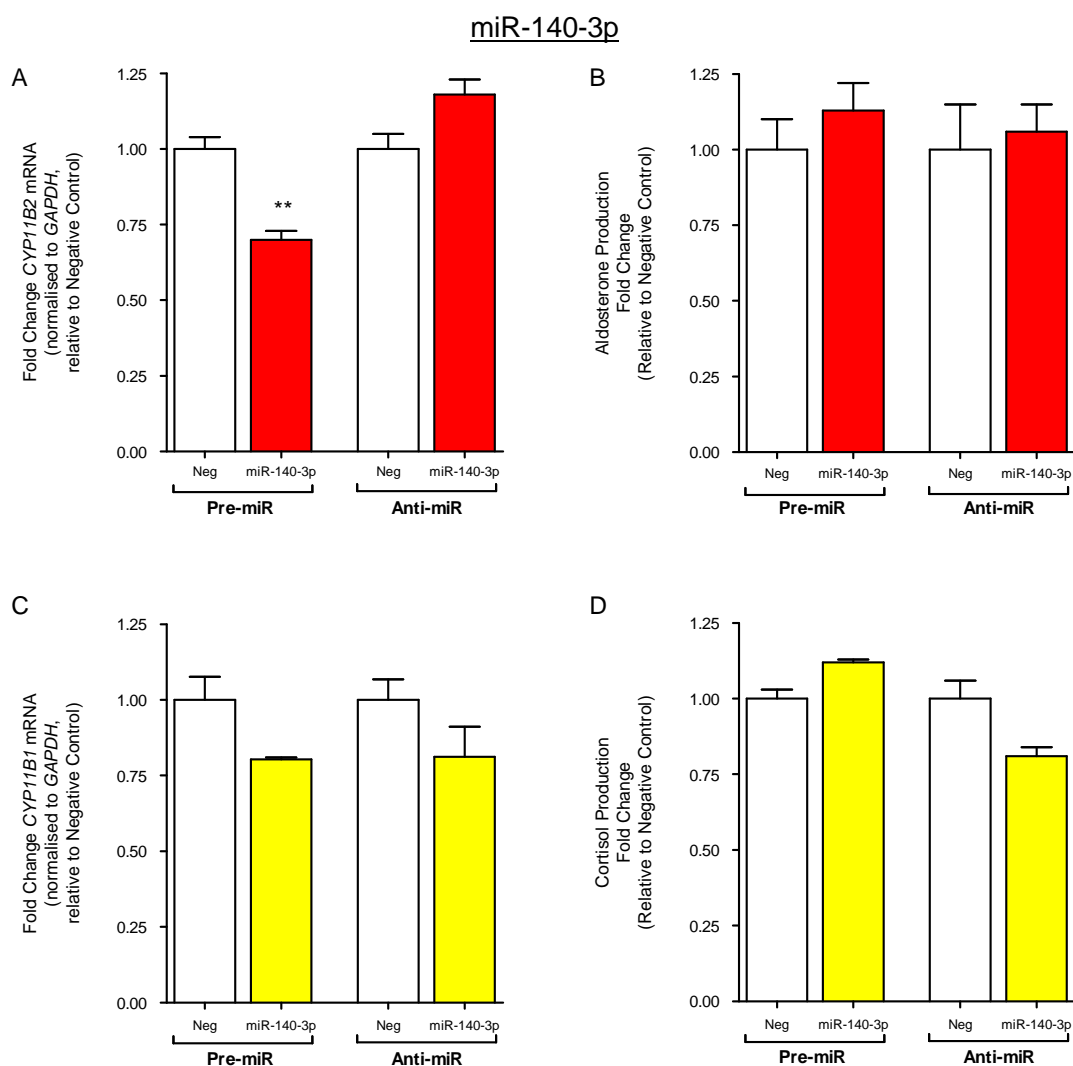


Figure 5-11. Assessment of miR-140-3p Effect on *CYP11B1* and *CYP11B2* mRNA Levels and on Steroid Production.

H295R cells were transfected with miR-140-3p Pre-miR™, miR-140-3p Anti-miR™ or with a scrambled negative control (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* (A) and *CYP11B1* (C) mRNA were measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The levels of aldosterone (B) and cortisol (D) secretion were measured by LC:MS/MS. Results represent the mean of one biological experiment performed in triplicate; error bars represent standard error of the mean (SEM). ** $p < 0.01$ compared to negative control.

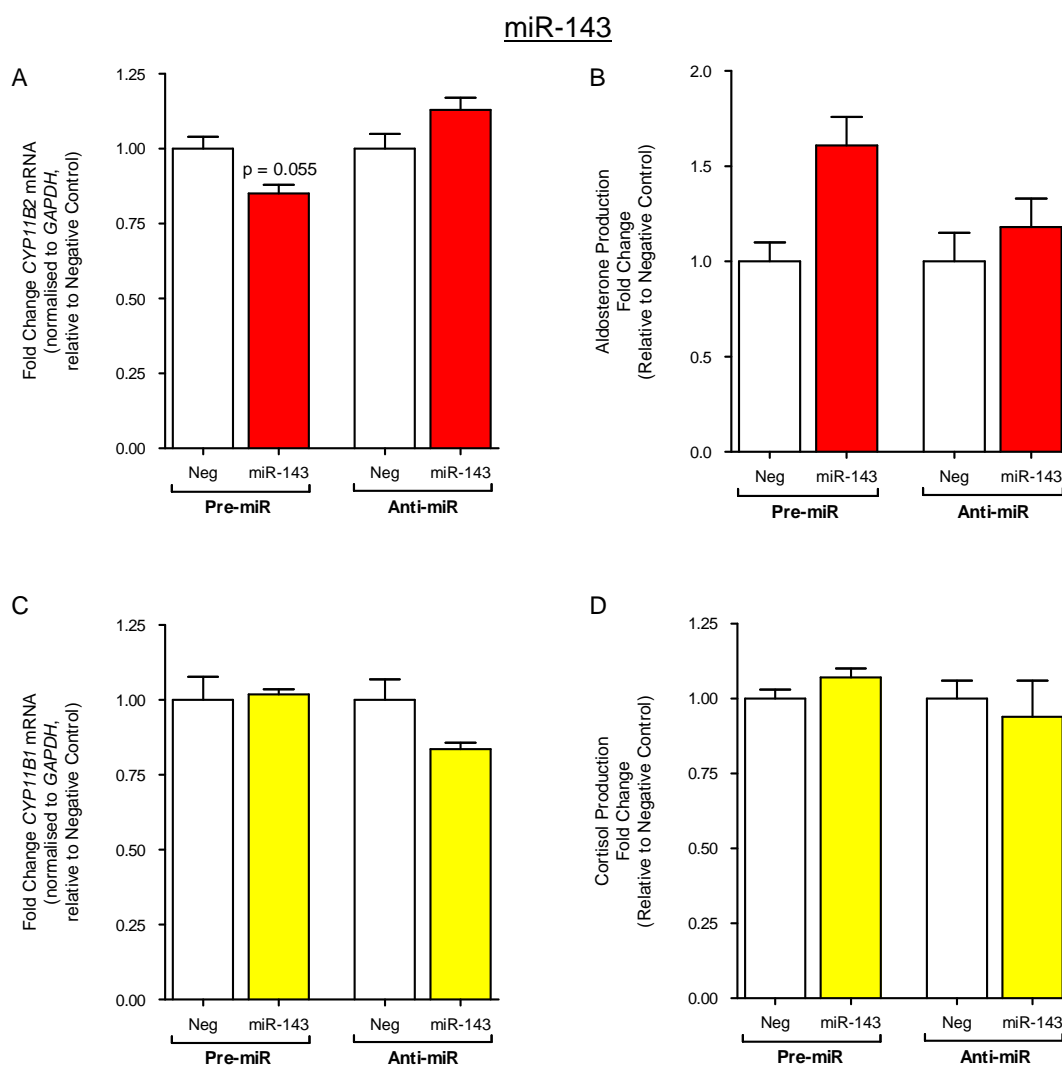


Figure 5-12. Assessment of miR-143 Effect on *CYP11B1* and *CYP11B2* mRNA Levels and on Steroid Production.

H295R cells were transfected with miR-143 Pre-miR™, miR-143 Anti-miR™ or with a scrambled negative control (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* (A) and *CYP11B1* (C) mRNA were measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The levels of aldosterone (B) and cortisol (D) secretion were measured by LC:MS/MS. Results represent the mean of one biological experiment performed in triplicate; error bars represent standard error of the mean (SEM). Where appropriate, p-values are as stated and are relative to negative control values.

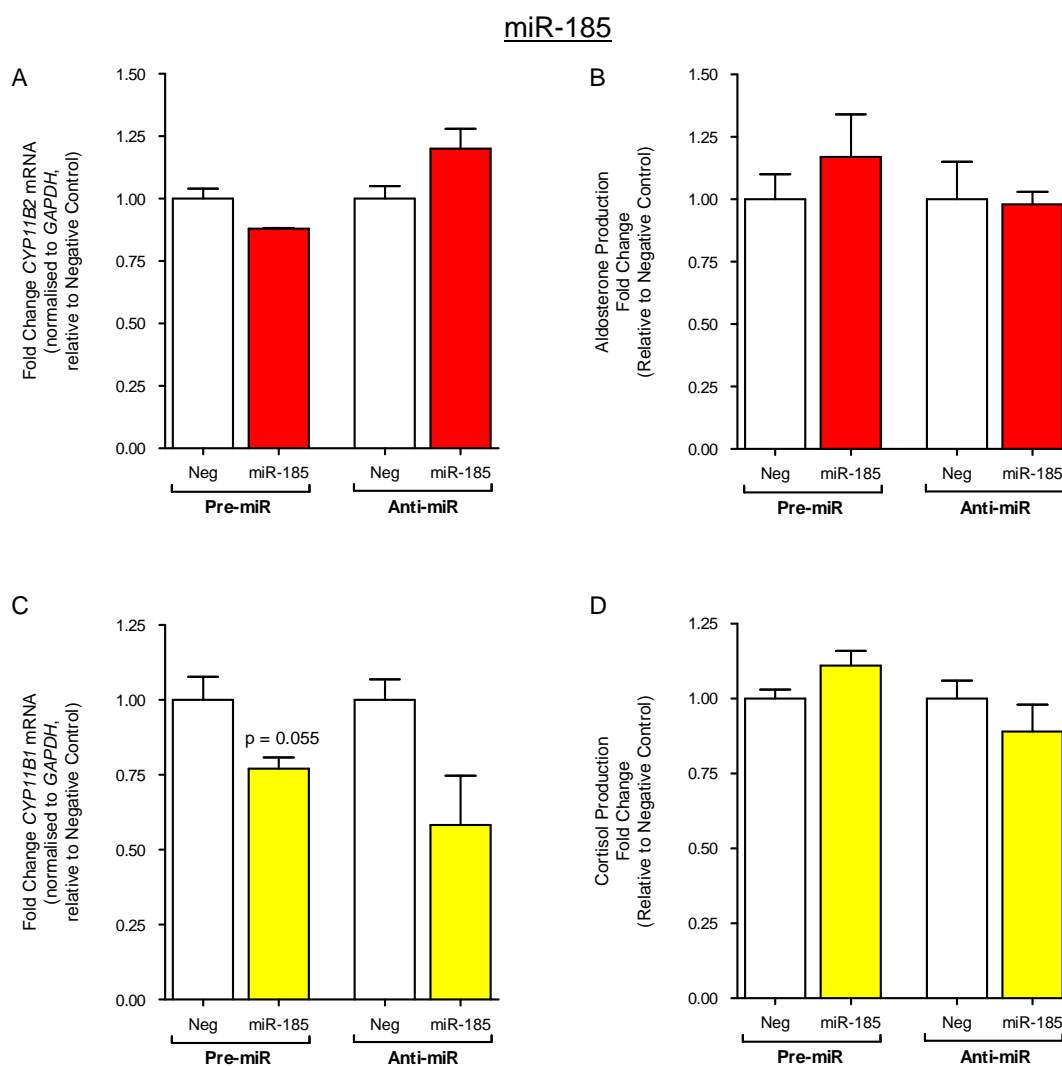


Figure 5-13. Assessment of miR-185 Effect on *CYP11B1* and *CYP11B2* mRNA Levels and on Steroid Production.

H295R cells were transfected with miR-185 Pre-miR™, miR-185 Anti-miR™ or with a scrambled negative control (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* (A) and *CYP11B1* (C) mRNA were measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The levels of aldosterone (B) and cortisol (D) secretion were measured by LC:MS/MS. Results represent the mean of one biological experiment performed in triplicate; error bars represent standard error of the mean (SEM). Where appropriate, p-values are as stated and are relative to negative control values.

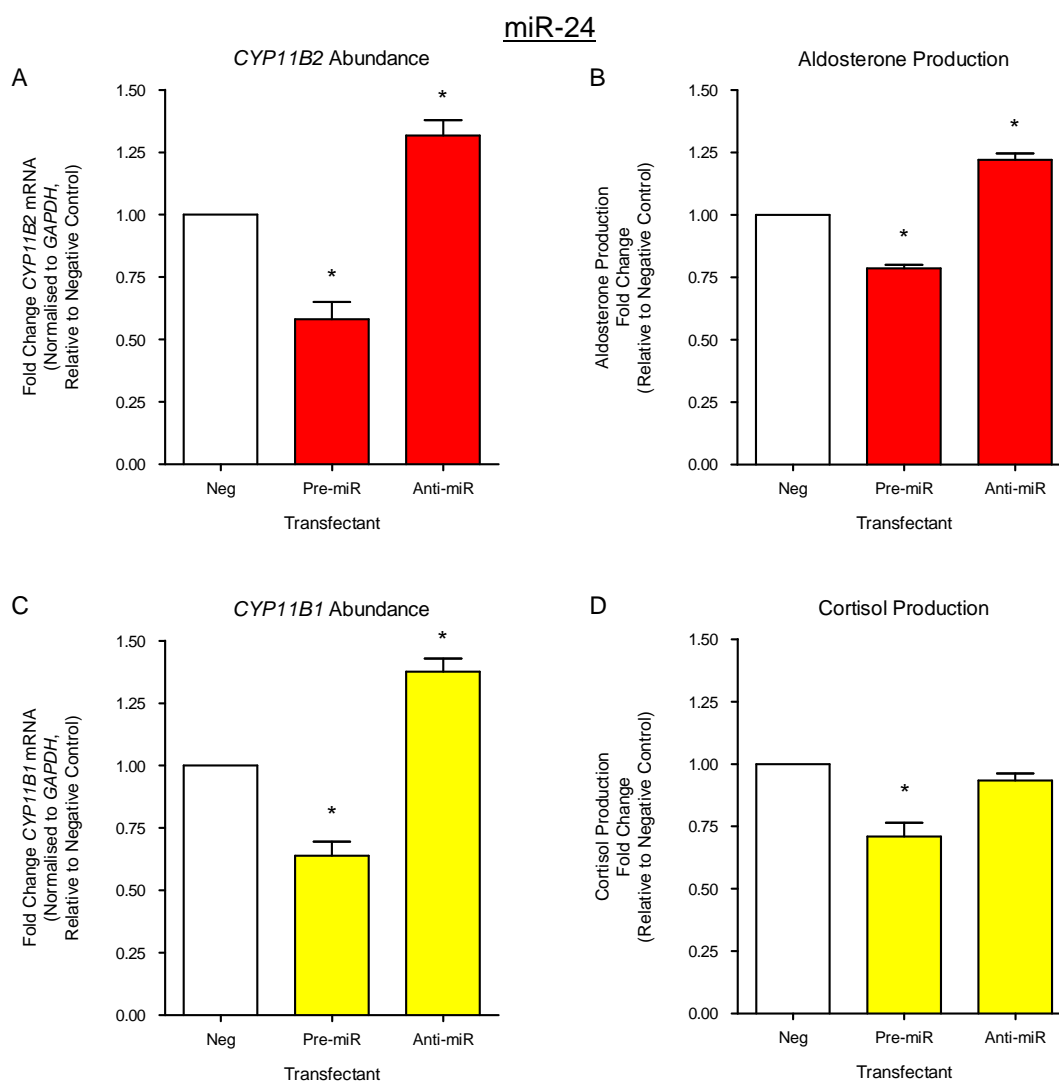


Figure 5-14. Assessment of miR-24 Effect on *CYP11B1* and *CYP11B2* mRNA Levels and on Steroid Production.

H295R cells were transfected with miR-24 Pre-miR™, miR-24 Anti-miR™ or with a scrambled negative control (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* (A) and *CYP11B1* (C) mRNA were measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The levels of aldosterone (B) and cortisol (D) secretion were measured by LC:MS/MS. Results represent the mean of three biological experiments performed in triplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$ compared to negative control.

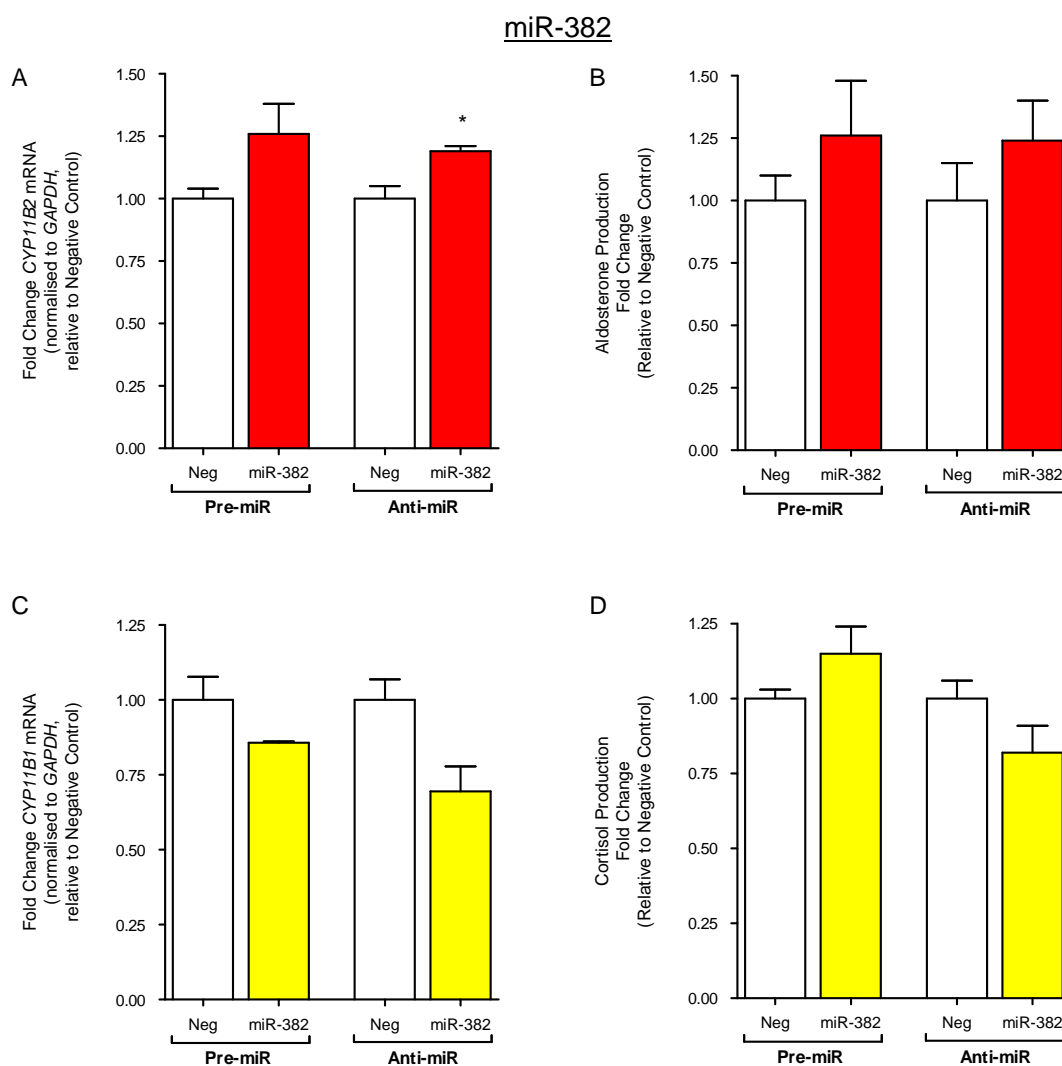


Figure 5-15. Assessment of miR-382 Effect on *CYP11B1* and *CYP11B2* mRNA Levels and on Steroid Production.

H295R cells were transfected with miR-382 Pre-miR™, miR-382 Anti-miR™ or with a scrambled negative control (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* (A) and *CYP11B1* (C) mRNA were measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The levels of aldosterone (B) and cortisol (D) secretion were measured by LC:MS/MS. Results represent the mean of one biological experiment performed in triplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$ compared to negative control.

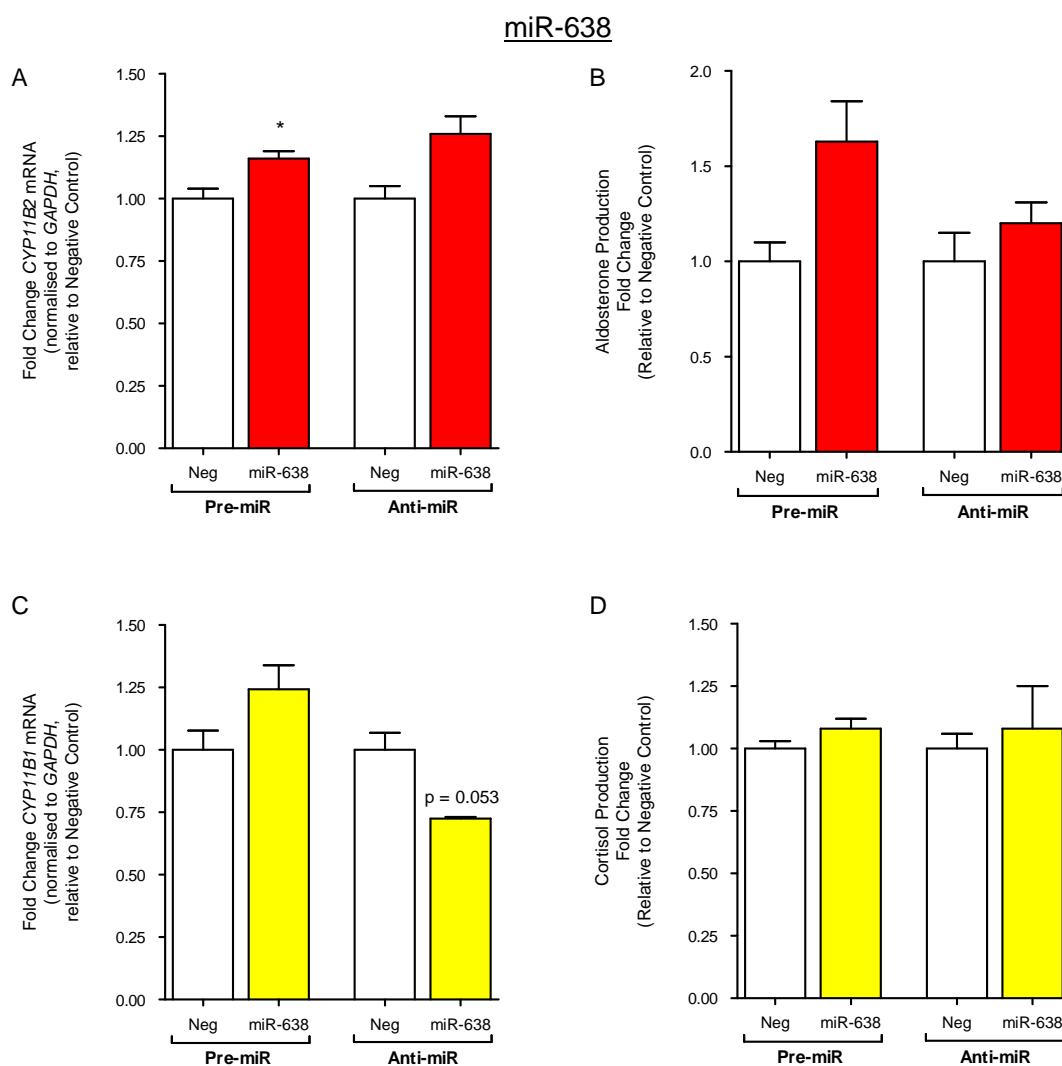


Figure 5-16. Assessment of miR-638 Effect on *CYP11B1* and *CYP11B2* mRNA Levels and on Steroid Production.

H295R cells were transfected with miR-638 Pre-miR™, miR-638 Anti-miR™ or with a scrambled negative control (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* (A) and *CYP11B1* (C) mRNA were measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The levels of aldosterone (B) and cortisol (D) secretion were measured by LC:MS/MS. Results represent the mean of one biological experiment performed in triplicate; error bars represent standard error of the mean (SEM). Where appropriate p values are stated, or * represents $p < 0.05$ relative to negative control.

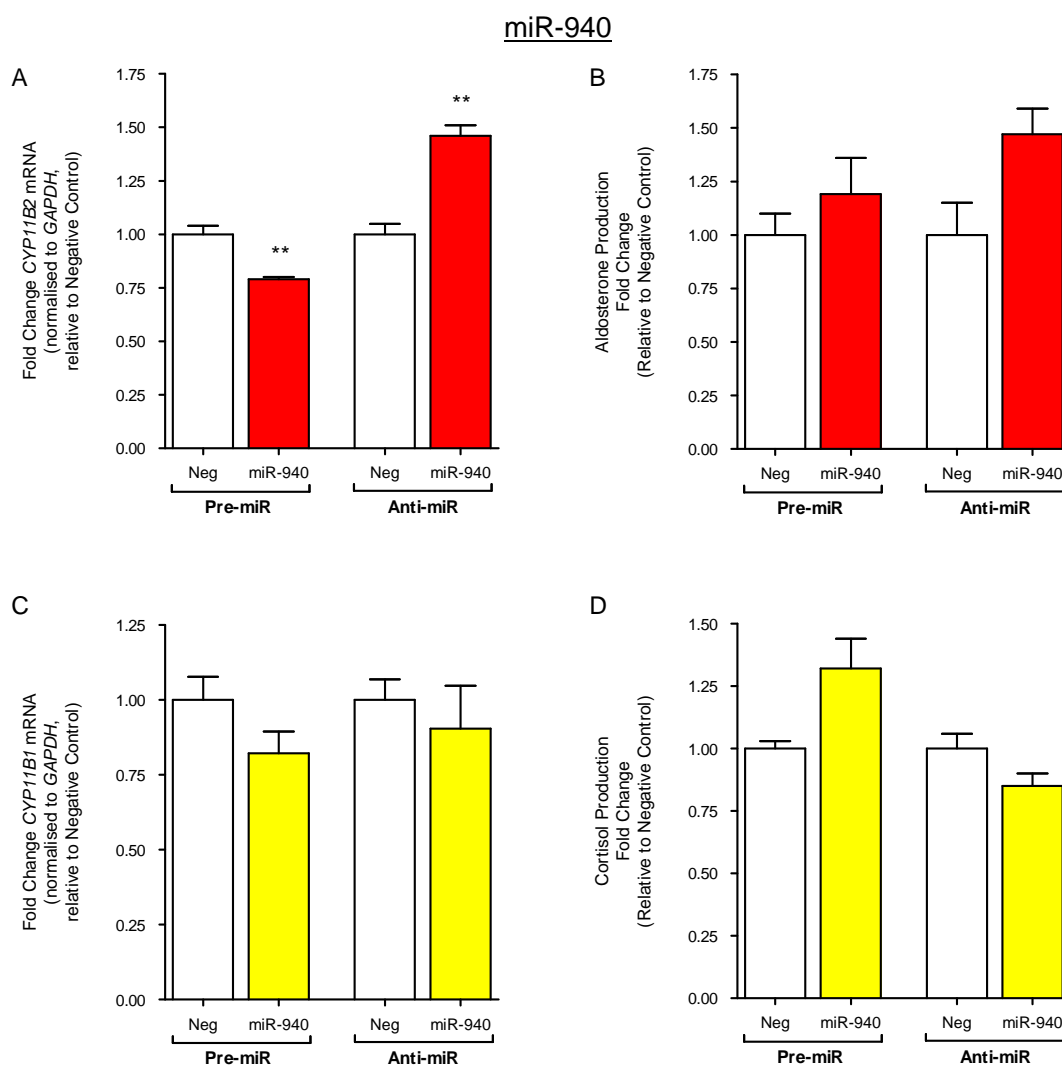


Figure 5-17. Assessment of miR-940 Effect on *CYP11B1* and *CYP11B2* mRNA Levels and on Steroid Production.

H295R cells were transfected with miR-940 Pre-miR™, miR-940 Anti-miR™ or with a scrambled negative control (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* (A) and *CYP11B1* (C) mRNA were measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The levels of aldosterone (B) and cortisol (D) secretion were measured by LC:MS/MS. Results represent the mean of one biological experiment performed in triplicate; error bars represent standard error of the mean (SEM). Where appropriate p values are stated, or ** represents $p < 0.01$ relative to negative control.

5.4.7 Effect of *CYP11B2*-Specific miRNAs on *CYP11B2* mRNA Abundance.

The results from the pEZX reporter construct studies described earlier in this chapter supported a role for a miR-125a-5p and miR-125b binding site on the 3'UTR of *CYP11B2*. To test this further, the Pre-miR™ or Anti-miR™ molecules corresponding to these miRNAs were transfected into H295R cells. The results for three independent biological replicate experiments are shown in Figure 5-18.

Firstly, results confirm that the action of miR-125a-5p on *CYP11B2* is typical of that exerted by miRNAs, with increasing miRNA levels leading to decreased *CYP11B2* mRNA abundance (0.70 ± 0.001 fold, $p = 0.004$) and decreasing miRNA levels leading to significant increase in *CYP11B2* mRNA abundance (1.53 ± 0.05 fold, $p = 0.009$, Figure 5-18A). Similarly, *CYP11B2* mRNA levels decreased to 0.66 ± 0.02 fold ($p = 0.033$) in Pre-miR™ transfected cells and increased by 1.58 ± 0.09 fold however, this failed to attain statistical significance ($p = 0.101$, Figure 5-18B).

A summary of all of the miRNA results detailed in this chapter is provided in Table 5-3 and Table 5-4.

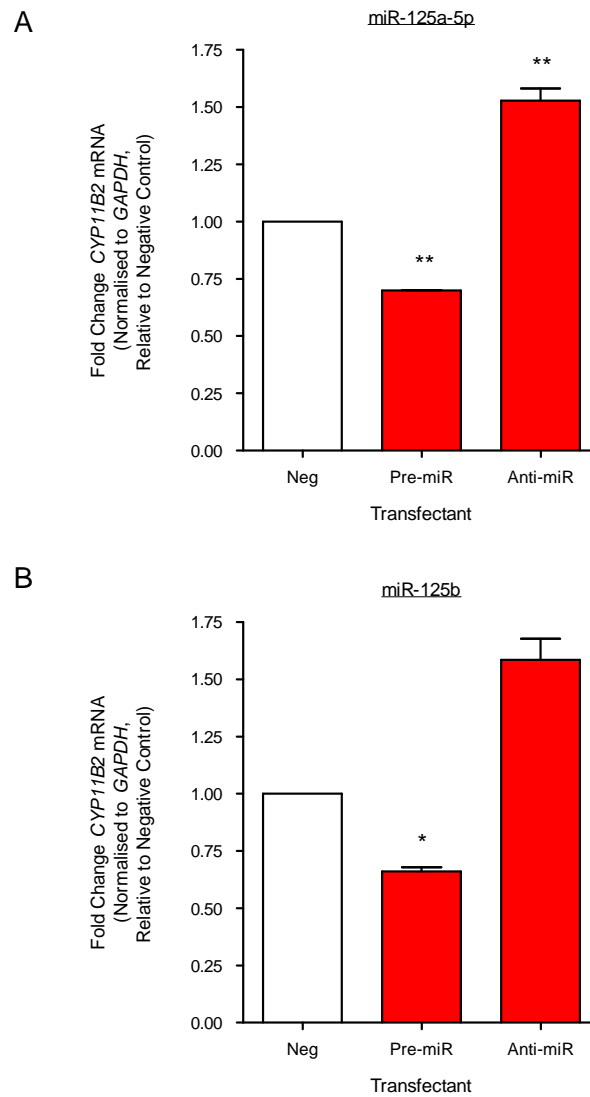


Figure 5-18. Assessment of miR-125a-5p and miR-125b Effect on *CYP11B2* mRNA Levels.

H295R cells were transfected with Pre-miR™ or Anti-miR™ molecules for miR-125a-5p (A), miR-125b (B) or with a scrambled negative control (Neg), at a final concentration of 50 nM. 48 Hours post-transfection, the abundance of *CYP11B2* mRNA was measured by qRT-PCR. Cycle threshold values were normalised to *GAPDH* mRNA and expressed relative to the control cells. The results represent the mean of two biological experiment performed in triplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.01$ compared to negative control.

Table 5-3. Summary of miRNA Target-Site Validation Experiments for the *CYP11B1* Gene.

| miRNA | <i>Seed Site</i> | 3'UTR | | mRNA | | Cortisol | |
|------------|------------------|---------|----------|---------|----------|----------|----------|
| | | Pre-miR | Anti-miR | Pre-miR | Anti-miR | Pre-miR | Anti-miR |
| miR-10a | 7mer-1A | ↔ | ↔ | - | - | - | - |
| miR-10b | 7mer-1A | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| miR-140-3p | 7mer-m8 | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| miR-143 | 7mer-1A | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| miR-185 | 7mer-m8 | ↓ | ↔ | ↓ | ↔ | ↔ | ↔ |
| miR-22 | 7mer-m8 | ↔ | ↔ | - | - | - | - |
| miR-24 | Offset 6mer | ↔ | ↑ | ↓ | ↑ | ↓ | ↔ |
| miR-34a | 7mer-m8 | ↔ | ↔ | - | - | - | - |
| miR-382 | Offset 6mer | ↓ | ↑ | ↔ | ↔ | ↔ | ↔ |
| miR-423-5p | Offset 6mer | ↑ | ↔ | - | - | - | - |
| miR-638 | 7mer-1A | ↔ | ↑ | ↔ | ↔ | ↔ | ↔ |
| miR-940 | 8mer | ↓ | ↔ | ↔ | ↔ | ↔ | ↔ |

Dashes (-): unmeasured parameters; unchanged arrows (↔): non-statistically significant changes.

Table 5-4. Summary of miRNA Target-Site Validation Experiments for the *CYP11B2* Gene.

| miRNA | Seed Site | 3'UTR | | mRNA | | Aldosterone | |
|-------------|-------------|---------|----------|---------|----------|-------------|----------|
| | | Pre-miR | Anti-miR | Pre-miR | Anti-miR | Pre-miR | Anti-miR |
| miR-10a | 7mer-1A | ↔ | ↔ | - | - | - | - |
| miR-10b | 7mer-1A | ↔ | ↔ | ↔ | ↔ | ↔ | ↔ |
| miR-140-3p | 7mer-m8 | ↔ | ↔ | ↓ | ↔ | ↔ | ↔ |
| miR-143 | 7mer-1A | ↓ | ↑ | ↓ | ↔ | ↔ | ↔ |
| miR-185 | 7mer-m8 | ↓ | ↑ | ↔ | ↔ | ↔ | ↔ |
| miR-22 | 7mer-m8 | ↑ | ↔ | - | - | - | - |
| miR-24 | Offset 6mer | ↔ | ↑ | ↓ | ↑ | ↓ | ↑ |
| miR-34a | 7mer-m8 | ↓ | ↔ | - | - | - | - |
| miR-382 | - | ↓ | ↔ | ↔ | ↑ | ↔ | ↔ |
| miR-423-5p | - | ↑ | ↔ | - | - | - | - |
| miR-638 | 7mer-1A | ↔ | ↔ | ↑ | ↔ | ↔ | ↔ |
| miR-940 | 8mer | ↓ | ↔ | ↓ | ↑ | ↔ | ↔ |
| miR-125a-5p | 8mer | ↔ | ↑ | ↓ | ↑ | - | - |
| miR-125b | 8mer | ↓ | ↑ | ↓ | ↔ | - | - |
| miR-134 | 7mer-m8 | ↔ | ↔ | - | - | - | - |
| miR- 495 | 7mer-m8 | ↔ | ↔ | - | - | - | - |

Dashes (-): unmeasured parameters; unchanged arrows (↔): non-statistically significant changes.

5.4.8 Investigating miRNA Clustered with miR-24.

The above results suggest a role for miR-24 in regulating the *CYP11B1* and *CYP11B2* mRNA levels and in steroid production. miR-24 is transcribed in an intronic cluster from a chromosomal location on chromosome 9, *C9orf3*, along with two other miRNA, miR-23b and miR-27b. These miRNAs are also expressed in the adrenal gland (Figure 4-6), with miR-23b predicted to bind the *CYP11B1* 3'UTR; miR-27b was not predicted to bind *CYP11B1* or *CYP11B2*. To test whether the individual components of this cluster act together to regulate the *CYP11B1* or *CYP11B2* genes, miR-23b and miR-27b were analysed using the pEZX reporter constructs in HeLa cells. The results of three independent biological experiments are shown in Figure 5-19.

Neither of these other two clustered miRNAs bind to and regulate *CYP11B1* or *CYP11B2* by canonical miRNA action. However, they do appear to be altering the luciferase activity of the reporter constructs; in miR-23b Anti-miR™ transfected cells, there was no significant change in luciferase activity (Figure 5-19A and B) but there was a significant increase in luciferase activity with the miR-23b Pre-miR™ transfected cells for both the pEZX-B2 ($226.10 \pm 29.29\%$; $p = 0.049$, Figure 5-19A) and pEZX-B1 reporter construct ($144.00 \pm 5.87\%$; $p = 0.017$, Figure 5-19B).

A similar trend was observed for miR-27b; the luciferase activity in Anti-miR™ transfected cells was not significantly different in pEZX-B2 cells and was marginally decreased in pEZX-B1 cells ($83.31 \pm 0.10\%$). This was statistically significant ($p = 0.003$) largely due to the small error associated with these results (Figure 5-19C and D). Furthermore, Pre-miR™ transfection increased the luciferase activity of both constructs: pEZX-B2 to $159.70 \pm 6.78\%$ ($p = 0.013$) and pEZX-B1 to $167.20 \pm 19.22\%$ ($p = 0.177$). While this result is not statistically significant, it is an increase of large magnitude (Figure 5-19C and D). The results for miR-24 are presented again in Figure 5-19E and F for comparison.

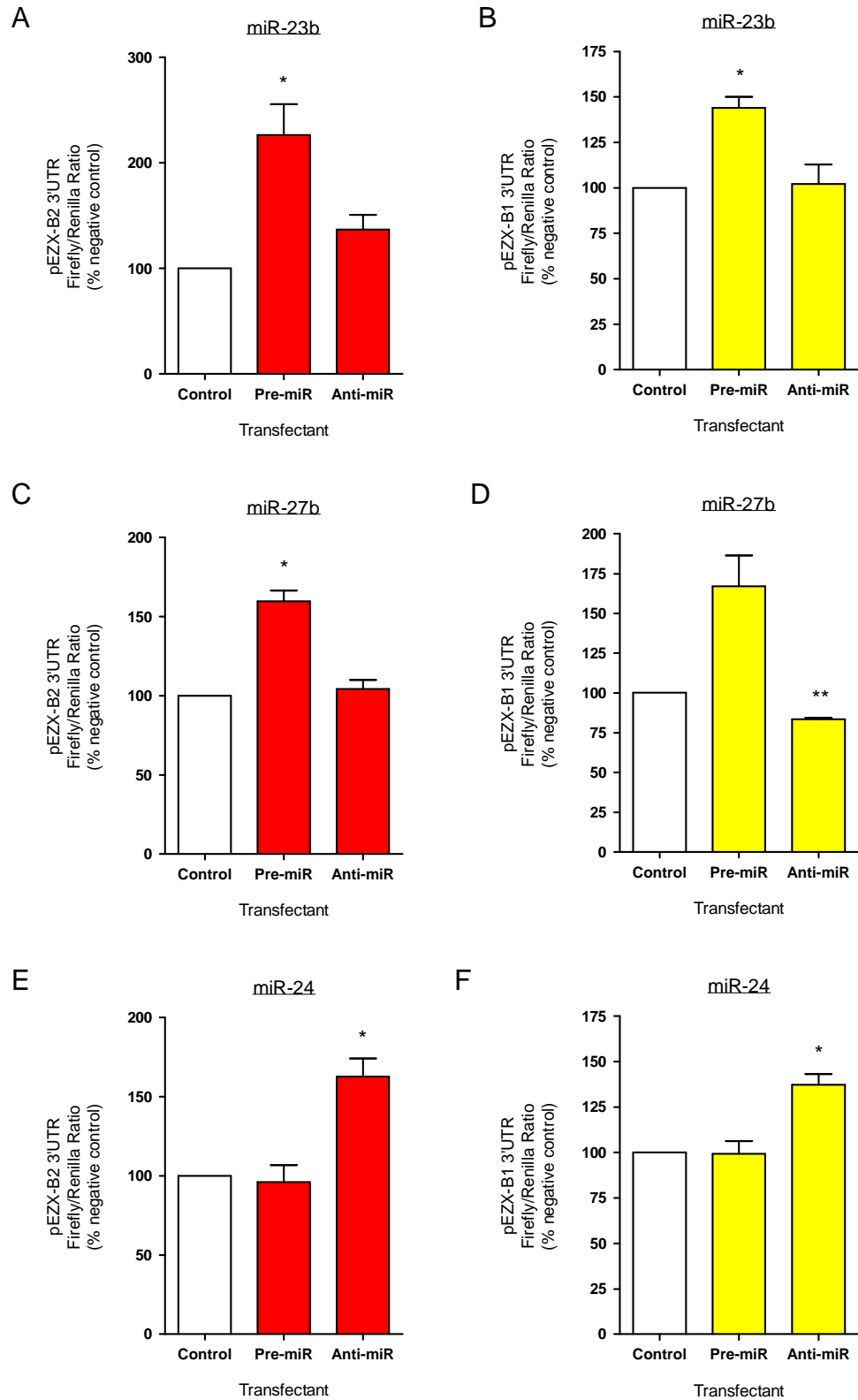


Figure 5-19. *CYP11B2* and *CYP11B1* 3'UTR Binding of miR-24 Cluster miRNAs.

HeLa cells were co-transfected with the pEZX-B1 (A, C, E) or pEZX-B2 (B, D, F) construct and Pre-miR™ or Anti-miR™ molecules for miR-23b (A-B), miR-27b (C-D) or miR-24 (E-F). Firefly and renilla luciferase were measured 48 hours post-transfection using the Dual Luciferase Reporter Assay System. To normalise for transfection efficiency, the ratio of firefly to renilla luminescence was calculated and expressed as a percentage of the negative scrambled control. Results represent the mean of at least three independent biological experiments, performed in quadruplicate; error bars represent standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.01$.

5.4.9 Investigation of the miRNA Binding Sites of miR-24, miR-125a-5p and miR-125b.

In addition to other findings, this chapter has identified a regulatory effect of miR-24 on both the *CYP11B1* and *CYP11B2* 3'UTRs and of miR-125a-5p and miR-125b on the *CYP11B2*. The sequence of the 3'UTR binding site and the mature miRNA were entered into in the RNAHybrid Secondary Structure Software, which has been specifically adapted to predict secondary structure of miRNA:mRNA interactions and to give greater detail regarding these binding sites (Kruger and Rehmsmeier, 2006).

miR-24 was predicted to target the *CYP11B2* gene by only one database, miRviewer, which also predicted the same site on the *CYP11B1* gene (Table 4-9 and Table 4-10); this site is an offset 6mer site spanning bases 489 to 502 of the 3'UTR (Figure 5-20A) with a predicted minimum free energy (MFE) of -22.1 kcal/mol. A second miR-24 site was predicted by microRNAs.org at bases 1206 to 1228 of the *CYP11B1* 3'UTR (Table 4-10). This site is a 7mer-m8 site, but with a mismatch at nucleotide number 6 (Figure 5-1B); the MFE for this predicted pairing is -27.3 kcal/mol.

Two databases predicted miR-125a-5p and miR-125b to bind to *CYP11B2* 3'UTR. Each was predicted to have the highest complementarity in the seed region (8mer) and furthermore, the region of the 3'UTR to which they bind was the same for both miRNAs (bases 1,200 to 1,223). The predicted secondary structure for both miRNA:mRNA interactions are shown in Figure 5-21 with the MFE for miR-125a-5p being -24.0 kcal/mol, and -28.0 kcal/mol for miR-125b.

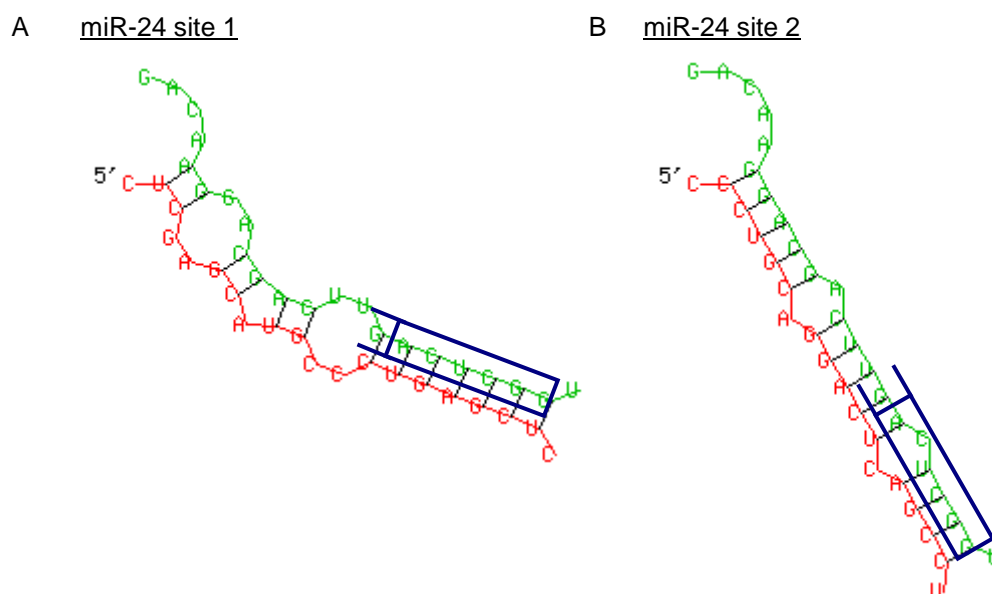


Figure 5-20. Predicted Secondary Structure of miR-24 Target Sites in the *CYP11B1* and *CYP11B2* 3'UTRs.

Secondary structure and base-complementarity of miR-24 (green) binding to 3'UTR of *CYP11B1* or *CYP11B2* (red). Blue boxes indicate the 6mer seed region and extensions indicate additional target recognition feature: complementary base-pairing and the 8th nucleotide. Site 1 (A) represents the binding of miR-24 to both the *CYP11B1* and *CYP11B2* 3'UTR. Site 2 (B) only exists in the *CYP11B1* 3'UTR. Structures predicted by RNAHybrid (Kruger and Rehmsmeier, 2006)

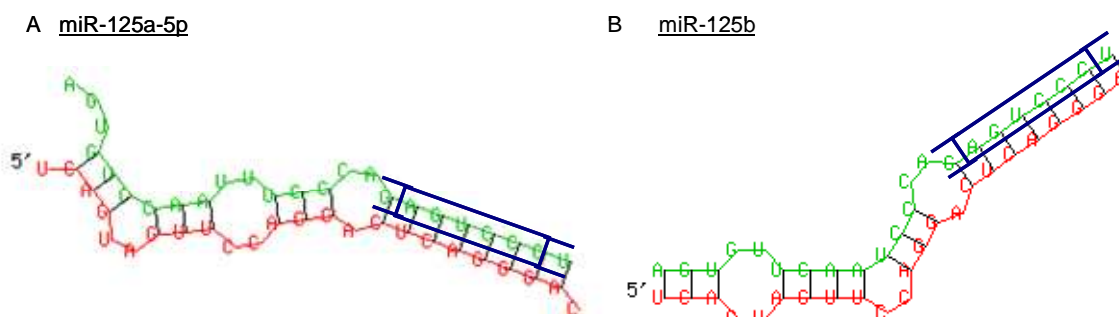


Figure 5-21. Predicted Secondary Structure of miR-125a-5p and miR-125b Target Sites in the *CYP11B2* 3'UTR.

Secondary structure and base-complementarity of miR-125a-5p (A) and miR-125b (B) (green) binding to the *CYP11B2* 3'UTR (red). Blue boxes indicate the 6mer seed region and extensions indicate additional target recognition features: an adenosine base at nucleotide 1 on the 3'UTR and the complementary base-pairing and the 8th nucleotide. Structures predicted by RNAHybrid (Kruger and Rehmsmeier, 2006).

5.5 Discussion

The experiments presented in this chapter investigated which of the miRNA target sites in the 3'UTR of *CYP11B1* and *CYP11B2* as predicted in Chapter 4 are regulated by the adrenal miRNAs identified in Chapter 3. Two primary methods were employed in the present chapter: 3'UTR reporter construct studies, and investigation of gain and loss of miRNA function in an adrenocortical cell line. The merits of these methods and the findings of this chapter, including the identification for the first time of miRNAs which directly regulate the *CYP11B1* and/or the *CYP11B2* genes, will be discussed below.

The first stage of miRNA target site validation utilised a 3'UTR reporter plasmid, which coupled a viral promoter-driven firefly luciferase coding region to the full length 3'UTR of the either *CYP11B1* or *CYP11B2* gene. This plasmid also contained a separate renilla luciferase reporter gene for the assessment of transfection efficiency. It was, therefore, not necessary to co-transfect a separate control reporter plasmid, which may have caused transfection efficiency problems, particularly as small molecules were also required to be transfected. By inserting the full length 3'UTR, rather than smaller sections that only encompass the predicted miRNA-binding region, the results of these experiments give a true reflection of miRNA:mRNA interaction and will, to a degree, account for the secondary structure present in this region (Rehmsmeier *et al.*, 2004). The HeLa cell line was used to evaluate miRNA binding; this was chosen as it is a well established cell line that does not change dramatically over time, is easy to transfect, has been used in other miRNA target site validation experiments and does not endogenously express the *CYP11B1* or *CYP11B2* genes, so miRNA target site competition would not be a problem for assessing miRNA action (Lim *et al.*, 2005).

The final experimental tools used in this chapter were the Pre-miR[™] and Anti-miR[™] molecules, which were purchased from Applied Biosystems. These are an established tool for use in miRNA investigation (Cheng *et al.*, 2005); similar types of molecule are available from other suppliers. The molecules are single stranded, so there is no risk of passenger strand expression and they are highly specific, which is important because miRNAs often only differ by one or two

bases and, if specificity is not high, there may be off-target effects. Furthermore, as shown in this chapter, the actions of either the Pre-miR™ or Anti-miR™ molecules can be directly assessed by measuring the levels of mature miRNAs post-transfection. This chapter has shown both that Pre-miR™ and Anti-miR™ were highly effective at modulating the levels of specific miRNAs. The change in miRNA levels were very large and probably not representative of the change observed in physiological circumstances however, as is often the case in *in vitro* model systems, a supraphysiological change is required to investigate a pathway. Moreover, the degree of change measured for several miRNAs was vastly different yet, in all cases the increase in miRNA expression would have been sufficient to exert a measurable level of regulation. These molecules offer the best method to consistently modulate the levels of a specific miRNA *in vitro* and are suitable for measuring changes in mRNA, as shown by changes in *PTK9* or *HMG2* mRNA abundance.

This chapter aimed to test the actions of the sixteen miRNAs predicted to have binding sites in the *CYP11B2* 3'UTR; twelve of these miRNAs also have a predicted binding site in *CYP11B1* so they were also tested for regulation that gene. During the course of this project, the bioinformatic target site predictions have been updated and now include two additional miRNAs that are predicted to bind to both genes: miR-28-5p and miR-34c-3p. Unfortunately, these miRNA were not investigated in this project and will need to be studied at a later date. Furthermore, another two miRNAs, miR-382 and miR-423-5p, are now only predicted to bind *CYP11B1* although, they have been tested against the *CYP11B2* gene. The updated list of adrenal miRNAs predicted to bind *CYP11B1* and *CYP11B2* is given in Figure 4-8.

The first phase of testing involved modulating the level of miRNAs and evaluating the response using a 3'UTR reporter construct. The results for the *CYP11B1* 3'UTR construct show that seven miRNAs either do not bind to the 3'UTR, or bind and cause effects that are not typical of the repressive miRNA action. The other five miRNAs showed promising results, suggestive of 3'UTR binding and consistent with canonical miRNA action. The influence of these miRNAs on endogenous *CYP11B1* mRNA abundance and steroid production was tested in the H295R cell line. Except for the miR-24 experiments, the other miRNA experiments were performed one biological time, with three technical

replicates. The actions of three out of the five miRNAs (miR-382, miR-638 and miR-940) were not verified in H295R cells. miR-185 and miR-24 were the only two miRNAs for which the results indicated a regulatory role. The magnitude of miR-185 action was small and will require replication to verify and the action of miR-24 will be discussed below.

A similar strategy was adopted for testing sixteen miRNAs for *CYP11B2* regulation; eight miRNAs were ruled out after reporter construct studies. Increasing miR-34a levels in HeLa cells did indicate miR-34a binding of the *CYP11B2* 3'UTR, but this miRNA was not investigated further in this project. The mRNA or steroid results for miR-185 did not verify the miRNA regulation that was observed with the reporter construct. The *CYP11B2* mRNA showed a trend towards regulation by miR-143 but aldosterone production was not consistent with this; more experimental replicates are required to understand the role of miR-143. Similarly, the results from H295R cells did not substantiate the reporter construct results indicating a role for miR-382. Given that this miRNA does not possess a putative binding site in the 3'UTR, according to the latest predictions, then the results support the current bioinformatic information.

The results from both validation experiments support a regulatory role for four miRNAs acting on the *CYP11B2* gene; three were tested comprehensively and the implications of these will be discussed below. The other one was miR-940, which consistently demonstrated regulation of *CYP11B2*, including actions at the 3'UTR and modulation of mRNA abundance. Unfortunately, only one experiment in H295R cells was performed but, with further replicates, it would be anticipated that these results would be validated and that the levels of aldosterone would be modulated accordingly.

Of the four miRNAs with predicted sites in *CYP11B2*, (miR-134 and miR-495), did not bind to the 3'UTR of *CYP11B2* and modulate luciferase activity so were not verified by this study. However, the results suggest that the other two mRNAs, miR-125a-5p and miR-125b, are genuine regulators of *CYP11B2* mRNA; both miRNAs decreased mRNA abundance when over-expressed in H295R cells. Inhibiting miR-125a-5p also increased the levels of mRNA significantly. The reporter construct studies confirmed that this regulation occurs through binding of the 3'UTR. These miRNAs are from the same family and therefore share

similar sequences; they are predicted to bind to the same seed site on the *CYP11B2* 3'UTR. This target site was predicted by two databases and predicted to have the highest complementarity in the seed region, 8mer. The magnitude of change in mRNA abundance is equivalent to that previously observed and is consistent with other reported results from miRNA targets with only one seed site in a 3'UTR (Selbach *et al.*, 2008). The location of the target site is near to the Poly (A) tail of the 3'UTR; miRNAs with target sites located at the proximal or distal end of the 3'UTR are likely to more effectively target and cause repression than those targeting central regions (Grimson *et al.*, 2007). The secondary structure predicted for miR-125b has a lower MFE than that of miR-125a-5p, which may suggest that this miRNA interacts with the mRNA preferentially (Rehmsmeier *et al.*, 2004). The value of the MFE is comparable to the preferential binding sites identified for let-7 targeting of *lin-14*, a well-verified miRNA:mRNA interaction (Reinhart *et al.*, 2000; Rehmsmeier *et al.*, 2004).

These miRNAs are expressed in a wide range of tissue types (Lagos-Quintana *et al.*, 2002) and are transcribed from separate chromosomal locations: miR-125a-5p from chromosome 11, and miR-125b from chromosome 19. miR-125a is transcribed alongside two other miRNAs, let-7e and miR-99b, which are also expressed within the adrenal gland (Figure 4-6) but were not predicted to bind to the *CYP11B2* 3'UTR. However, in addition to miR-125a-5p regulating *CYP11B2*, these other miRNAs may regulate other genes, providing a novel adrenal regulatory network.

Due to their sequence similarity, miR-125a and miR-125b are frequently studied together. miR-125a-5p and miR-125b have both been shown to repress two members of the ERBB oncogene family (*ERBB2* (HER2) and *ERBB3* (HER3)). Targeting of these genes in a breast cancer cell line, by exogenous overexpression of miR-125a-5p or miR-125b, reduced cell growth and migration (Scott *et al.*, 2007). Furthermore, they have both been shown to regulate the levels of endothelin-1 (ET-1) in vascular endothelial cells by targeting preproET-1 at its 3'UTR (Lia *et al.*, 2010). Therefore, co-regulation of the *CYP11B2* gene by miR-125a-5p and miR-125b, as described in this chapter, is not unusual. The interplay between these miRNAs has not been established; it would be interesting to establish whether they act synergistically or competitively.

Increasing the levels of both of these miRNA in H295R cells or with a reporter construct may help to answer these questions.

Finally, miR-125b has been implicated in cardiac hypertrophy. It was found to be upregulated in a mouse model of cardiac hypertrophy, when compared with the hearts of control animals, although the authors failed to directly correlate increased expression of miR-125b with a target gene or pathway (van Rooij *et al.*, 2006). This miRNA has been implicated in regulation of cell proliferation and its expression is down regulated in a range of cancer tissues and cancer cell lines (Lee *et al.*, 2005; Iorio *et al.*, 2005). Therefore, in adrenal cells miR-125b may have target multiplicity and control several pathways.

The only miRNA which was identified in this chapter as a regulator of both *CYP11B1* and *CYP11B2* mRNA abundance and of steroid production was miR-24. This miRNA targets one site that is common to both genes: an offset 6mer site with an additional G:U wobble pairing at base 2, which can be tolerated in miRNA seed sites (Bartel, 2009). There is a further 7mer-m8 target site unique to the *CYP11B1* 3'UTR, although this has a 7mer-m8 with a mismatch in the seed region. However, this site also contains a large degree of base-pairing complementarity to the centre region and 3' end of the miRNA, which would most likely compensate for this mismatch (Shin *et al.*, 2010).

The reporter construct studies confirmed that inhibiting the levels of miR-24 caused an increase in luciferase activity. However, the corresponding decrease was not observed when miR-24 was over expressed. One possible explanation is that high endogenous levels of miR-24 in HeLa cells may already occupy the available miR-24 targets sites and repress the construct to a maximal level (Ritchie *et al.*, 2010). Nonetheless, experiments with the Anti-miR™ molecule confirm that miR-24 regulation of the *CYP11B1* and *CYP11B2* genes occurs at the 3'UTR and investigations in H295R cells were consistent with this, showing that miR-24 acts to regulate both of these genes in a canonical miRNA manner.

miR-24 is transcribed from two genomic locations, known as miR-24-1 (intronic region on Chromosome 9) and miR-24-2 (intergenic region on Chromosome 19). These miRNAs have identical mature sequences but different primary transcripts; therefore they have similar biological function but may have

different regulation and expression patterns (Chhabra *et al.*, 2010). Each miRNA is transcribed as part of a triplet cluster that includes miR-23a and miR-27a (cluster 2) or miR-23b and miR-27b (cluster 1). The sequences of the miR-23 and miR-27 families are not identical and vary by one or two bases, meaning their mRNA targets will be different. The members of the miR-24-1 cluster were also tested for their action on *CYP11B1* and *CYP11B2* (miR-23b has a putative binding site on *CYP11B1*), but neither miRNA demonstrated canonical miRNA action. Both caused an increase in luciferase activity when over expressed in HeLa cells but the meaning of this is unclear; miR-27b is not thought to have a site on either 3'UTR and miR-23b only on *CYP11B1*, so these miRNA may be having an indirect effect that subsequently increases the transcription of luciferase.

The implications of novel regulation by miR-24 are still to be identified. It may have a role in maintaining *CYP11B1* and *CYP11B2* mRNA levels at baseline levels, or changes in the level of miR-24, for example in response to AngII, may allow for increases in *CYP11B1* and *CYP11B2* expression, thus increasing aldosterone and/or cortisol production. The members of both of the miR-24 clusters have been reported to be dysregulated in several pathologies, including several types of cancer, although there does not appear to be a consistent pattern of expression, with the miRNA clusters sometimes being increased and, at other times, reduced in cancer samples (Volinia *et al.*, 2006; Saumet *et al.*, 2009).

Previous studies have identified a role for miR-24 in the regulation of several cellular pathways (Chhabra *et al.*, 2010). The action of miR-24 appears to be cell-type specific, as it has been reported to regulate opposing cell mechanisms, such as cell proliferation and cell apoptosis (Lal *et al.*, 2008; Qian *et al.*, 2011). The majority of validated targets for miR-24 are related to regulation of the cell cycle (Chhabra *et al.*, 2010), which may explain the observed differential expression in tumour tissue. However, in this chapter miR-24 has been shown to regulate *CYP11B1* and *CYP11B2* mRNA levels in normal conditions. Whether adrenal miR-24 has a dual function and contributes to adrenal plasticity remains to be seen.

The results presented in this chapter highlight some difficulties in assessing miRNA function. Because miRNAs are promiscuous, altering the levels of only one miRNA may not represent the true physiological situation. miRNAs are

hypothesised to only modulate mRNA levels by small amounts, which may be difficult to measure unless specific and sensitive methods, such as those employed in this chapter, are used. Moreover, the results highlight the shortcomings of bioinformatic miRNA target prediction algorithms: very few of the predicted miRNAs could be validated as the target of either gene by either of the two methods used here. However, the techniques chosen and developed in this chapter are frequently used to assess miRNA targeting and they were carefully verified to ensure a good level of accuracy. They proved successful at validating some miRNA target predictions and at discounting others. Other strengths of this chapter include the use of the H295R cell line; this is a human cell line which maintains basal steroid secretion. Assessing miRNA function in these cells is the best available model for studying steroid production in the human adrenal gland. Target validation could be taken further by mutating the seed region in the 3'UTR construct and assessing whether this disrupts miRNA function. Other more sophisticated techniques include HITS-CLIP (high throughput sequencing of cross-linking immunoprecipitation), which offers the advantage of assessing the action of endogenous miRNA on endogenous mRNA targets (Chi *et al.*, 2009). Further, modified versions of SILAC (stable isotope labelling with amino acids in cell culture), including pulsed SILAC, have been developed to monitor changes in novel protein production following miRNA transfection. This method allows for many targets of a single miRNA to be identified (Selbach *et al.*, 2008).

In summary, these studies have investigated putative miRNA binding sites in the *CYP11B1* and *CYP11B2* 3'UTRs. This chapter has disproved some of the bioinformatic prediction and found several miRNAs to be novel regulators of *CYP11B1* mRNA and cortisol production and/or of *CYP11B2* mRNA and aldosterone production. miR-24 is the best example of a miRNA regulating the expression of these genes in an adrenal cell line. Regulation of miR-24 expression and its function under stimulated or pathophysiological conditions may prove interesting in circumstances of altered adrenal function. In conclusion, the results presented in this chapter show for the first time that the *CYP11B1* and *CYP11B2* genes are directly regulated by miRNAs.

6 Altered Adrenal-miRNA Expression and Function.

6.1 Introduction

Altered miRNA expression and/or disrupted miRNA regulation have been implicated in the aetiology of several pathologies (Section 1.7.1 and 1.7.2); investigating the influence of miRNA regulation and expression on adrenal-related disease will be the focus of the studies in this chapter.

There are numerous ways in which miRNAs may impact on disease. For example, they may be important in disease initiation and progression, they may contribute the symptoms of a disease and, additionally, aberrant miRNA expression may occur as a result of the pathology itself. A single miRNA could contribute to more than one of the deleterious effects in a pathological state. For example, miRNAs have been reported to affect cell cycle regulation and vascularisation of tissue, and also have direct effects on cell-specific genes. Finally, because normal physiological miRNA regulation is required to moderate mRNA stability by only small amounts, then any change in miRNA abundance could be associated with subtle changes in cell function and phenotype, such as increased aldosterone production and small changes in blood pressure.

Both relative miRNA expression and target site sequence can influence miRNA-mediated regulation. These changes can be divided into *cis*-factors (such as polymorphisms at the genomic loci encoding miRNAs, epigenetic modifications and chromosomal alterations) or by *trans*-factors (e.g. polymorphisms that disrupt established miRNA target sites or create novel ones, or polymorphisms in the genes encoding proteins involved in miRNA processing and maturation) (Sethupathy and Collins, 2008; Ryan *et al.*, 2010). A specific example of a *cis*-acting polymorphism and a *trans*-acting polymorphism is given in Section 1.5.2.2 and Section 1.7.2, respectively.

The initial studies in this chapter aim to explore the role of miRNAs in the aetiology of aldosterone-producing adenoma (APA), one of the common types of primary aldosteronism (PA) (Young, 2007) (Section 1.4.1.1). The cause of these spontaneous masses is unknown but they occur more frequently in patients with the -344T *CYP11B2* promoter allele (Inglis *et al.*, 2001).

In addition to the miRNA expression data for the normal adrenal gland presented in Chapter 4, a small number of studies have investigated the role of miRNAs in adrenal samples of various origins (Tombol *et al.*, 2009; Soon *et al.*, 2009; Iliopoulos *et al.*, 2009; Bimpaki *et al.*, 2010; Schmitz *et al.*, 2011). However, only two investigated miRNA expression in adrenocortical adenoma and only a subset of these samples were classified as Conn's tissue (Schmidt *et al.*, 2003; Soon *et al.*, 2009). Moreover, the coverage of miRNAs and access to data presented in these studies are limited. Therefore, further studies were warranted. To this end, the miRNA expression profile of APA samples was determined by miRNA microarray, the data-set generated being comparable to the comprehensive normal adrenal gland miRNA expression profile (Chapter 4). Currently, the cause and regulation of increased *CYP11B2* expression in APAs is unknown (Gomez-Sanchez and Gomez-Sanchez, 2010); the information gained from performing this microarray experiment will be useful in the identification of miRNAs involved in neoplastic adrenal growth and will also, in conjunction with earlier studies presented in this thesis, contribute to our understanding of miRNA regulation of *CYP11B2* mRNA.

The latter studies in this chapter will be concerned with identifying genetic variation in the 3'UTR of the *CYP11B1* and *CYP11B2* genes and investigating how this may influence miRNA-binding. One hypothesis is that such polymorphisms may lie in miRNA binding sites and, hence, alter miRNA-mediated regulation of these genes under basal conditions, thereby leading to changes in the levels of corticosteroid production and contributing to the aetiology of essential hypertension.

6.2 Aims

The aims of this study were to identify whether miRNA-mediated regulation of corticosteroidogenic genes contribute to the development or the phenotype of adrenal pathology and to determine and investigate genetic variation in the 3'UTR of the *CYP11B1* and *CYP11B2* genes, to compare the miRNA expression in aldosterone-producing adenoma samples to that of the normal adrenal gland.

6.3 Methods

6.3.1 *miRNA Expression Profiling of Aldosterone-Producing Adenomas*

Total RNA was isolated from four formalin-fixed paraffin-embedded aldosterone-producing adenoma tissue samples (section 2.2.2) and quantified as described in Sections 2.2.4 and 2.2.5. Samples were shipped to the microarray provider (LC Sciences, Houston, Texas) and the array experiment was performed (Section 2.3). A background threshold cut-off of 500 arbitrary units was used, as recommended by the array chip manufacturer. miRNAs expressed at levels greater than this threshold were deemed to be expressed in the adrenal gland.

A cross-array normalisation was performed between normal adrenal and APA microarray experiments. The relative signal intensities of normal and APA samples were compared.

6.3.1.1 Statistical Analysis of Expression Data

The correlation of the microarray expression data and miRNA expression differences (Student's t-test) were tested using Prism 4.0 Graph Pad software. For all analyses, confidence intervals of 95% were used and $p < 0.05$ was required for statistical significance. Data are expressed as the mean \pm SEM.

6.3.2 *Genotyping of the 3'UTR of CYP11B1*

6.3.2.1 Subjects

Twenty-six normotensive patients participating in the North Glasgow Monitoring Trends and Determinants in Cardiovascular (MONICA IV) were selected for analysis (Fraser *et al.*, 1999). These subjects had previously been sequenced for two common genetic variants: the *CYP11B2* promoter -344 C/T SNP and the intron 2 conversion. The characteristics of these patients have previously been published (Barr *et al.*, 2007).

6.3.2.2 Sequence Analysis

The full-length *CYP11B1* 3'UTR (2014 base-pairs) was first amplified by PCR (Section 2.6.1), verified by gel electrophoresis (Section 2.6.2) and the products were then sequenced according to methods described in Section 2.7 using the primers in Table 6-1.

Table 6-1. Primers Used to Sequence the *CYP11B1* 3'UTR.

| Primer Name | Sequence (5'-3') | Direction |
|--------------|----------------------|------------|
| B1 ex9 seq1F | CAGGTGGAGACACTAACCCA | Sense |
| B1 ex9 seq3F | AGTCTCACATGTCCCTGTTC | Sense |
| B1 ex9 seq5F | AAGGACTCAGACGAGTTTTA | Sense |
| B1 ex9 seq7F | AAGAAAACGCCATAGACTGG | Sense |
| B1 ex9 R1 | ATGCTCTGCCCCTGCAGCTT | Anti-sense |

6.3.2.3 Statistical Analysis of Genetic Sequences

Sequencing was visualised using the SeqScape v. 2.1.1 software. Data taken from Ensembl release 66 (Feb 2012) using genotype data from the 1000 genome CEU population; low coverage panel was analysed using Haploview v. 4.2 Software (Barrett *et al.*, 2005). Genotype distribution was calculated by using the Hardy Weinberg equilibrium and LD calculated using D'.

6.4 Results

6.4.1 miRNA Expression Profiling of Aldosterone-Producing Adenoma Tissue.

The results of the microarray experiment are shown in Figure 6-1; the red line represents the expression cut-off of 500 AU, 62 miRNAs were expressed in APA tissue above the stated threshold. The mean expression level of these miRNAs is shown in Figure 6-2. The signal intensity for individual miRNAs was consistent between the samples.

To compare the relative expression levels of miRNAs in aldosterone-producing adenoma samples to those in the normal adrenal gland, the two miRNA microarrays were normalised using a global normalisation approach which allows for direct comparison of the two data-sets. This adjusted the signal intensities for the normal adrenal gland miRNA expression results so that the signal intensity of 67 miRNAs was greater than 500 AU following normalisation. Of these 51 were also expressed in the APA samples and 16 were not. Eleven miRNAs were only expressed in APA samples (Figure 6-3).

The signals from the normal and APA samples were highly correlated ($p < 0.0001$, r^2 0.825; purple dots, Figure 6-4). This correlation was maintained when only samples expressed above 500 AU were analysed ($p < 0.0001$, r^2 0.8703; red diamonds, Figure 6-4). 27 highly-expressed miRNAs are differentially expressed by greater than 2-fold (Figure 6-4). Table 6-2 lists the mean expression levels of all the miRNAs which differed significantly between samples types, including those which are classified as being expressed in only one tissue, as determined by microarray analysis.

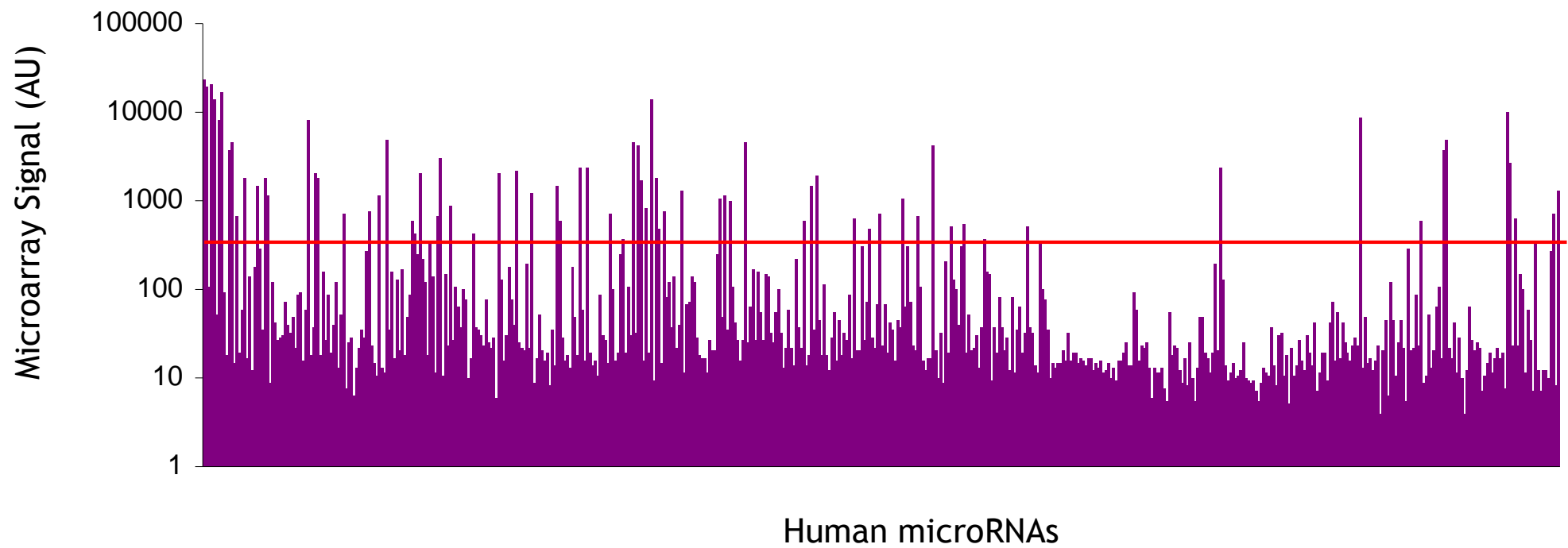


Figure 6-1. Aldosterone-Producing Adenoma miRNA Microarray Output.

The average normalised microarray expression signal of 728 human miRNAs (x-axis, in miRNA numerical order) in four aldosterone-producing adenoma samples. Red line represents the expression cut-off threshold of 500 Arbitrary Units (AU).

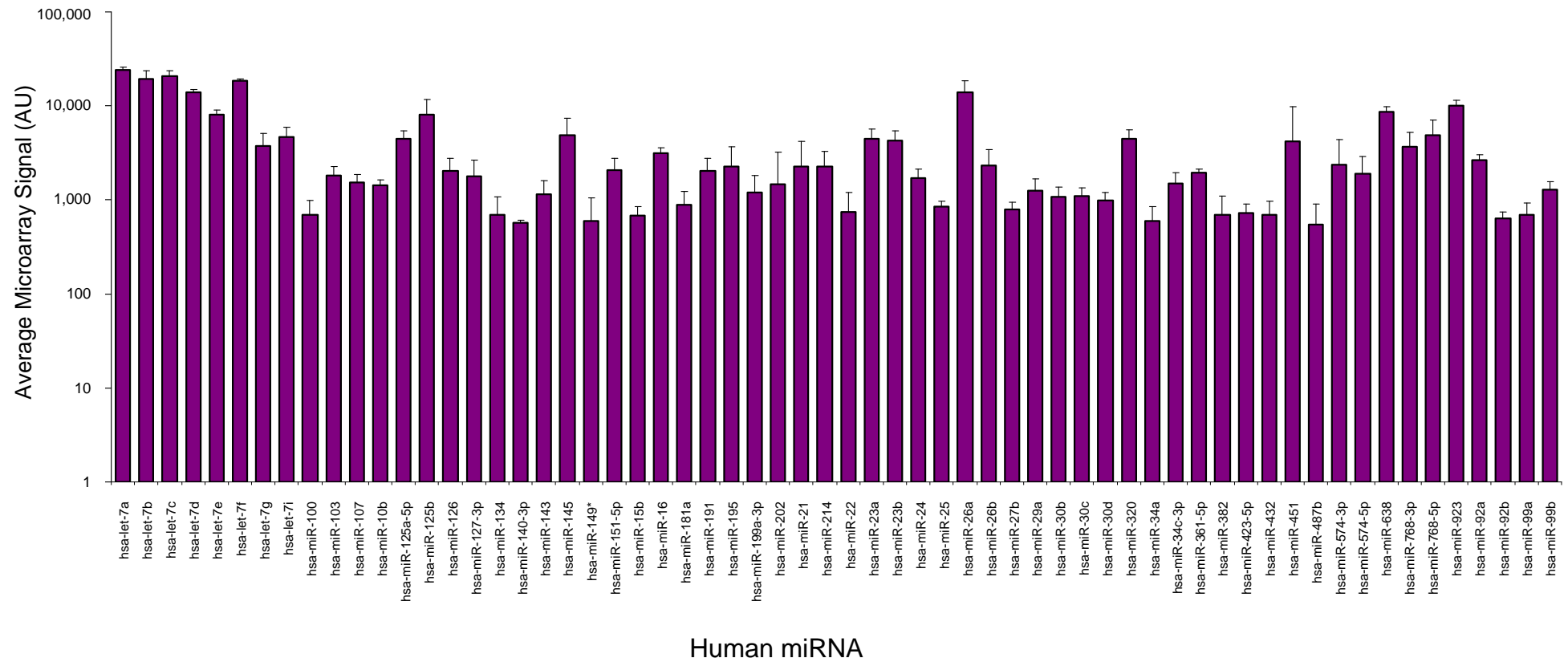


Figure 6-2. Average Microarray Signals of miRNAs Expressed in Aldosterone-Producing Adenoma.

The average normalised microarray expression signal of miRNAs with an expression level greater than 500 Arbitrary Units (AU). The mean of four tissues samples; error bars represent SEM.

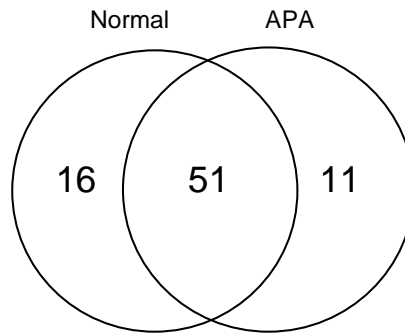


Figure 6-3. Number of miRNAs Expressed in Normal and APA Samples.

The number of miRNAs expressed in each sample type: normal (non-tumorous adrenal gland) and APA (aldosterone-producing adenoma) following miRNA microarray normalisation.

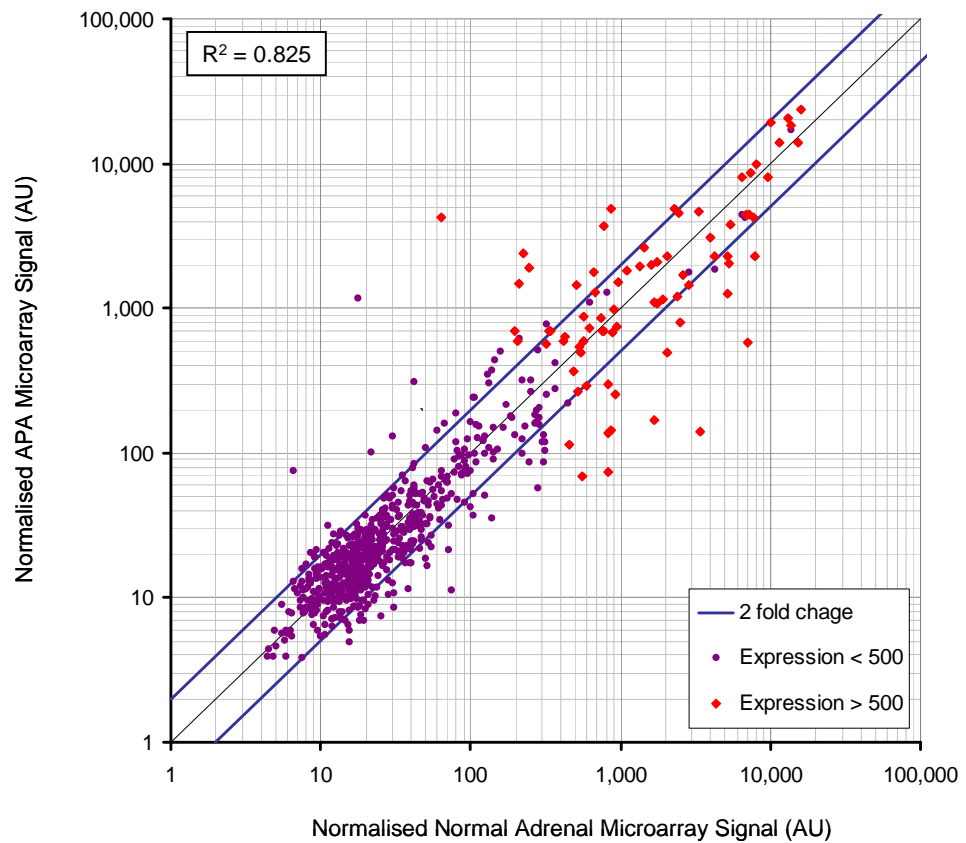


Figure 6-4. Correlation of miRNA Expression in Normal and APA Adrenal Tissue.

miRNA expression levels (purple: < 500 AU; red: >500AU) from normal adrenal gland (x-axis) and APA samples (y-axis) on log scales. Blue line indicates change of greater than 2-fold.

Table 6-2. Normalised Adrenal and APA Microarray Signals.

| miRNA | Normal Adrenal | | APA | | p-value |
|-------------|----------------|-------|----------|-------|---------|
| let-7a | 16,002 ± | 1,189 | 23,866 ± | 895 | < 0.01 |
| let-7b | 10,072 ± | 855 | 19,235 ± | 2,202 | < 0.01 |
| let-7c | 13,232 ± | 1,047 | 20,486 ± | 1,593 | < 0.01 |
| let-7d | 11,534 ± | 756 | 14,091 ± | 480 | < 0.05 |
| let-7f | 13,709 ± | 557 | 18,592 ± | 407 | < 0.01 |
| miR-103 | 1,109 ± | 138 | 1,813 ± | 217 | < 0.05 |
| miR-107 | 958 ± | 120 | 1,527 ± | 176 | < 0.05 |
| miR-10b | 2,835 ± | 374 | 1,438 ± | 107 | < 0.05 |
| miR-125a-5p | 6,919 ± | 715 | 4,475 ± | 466 | < 0.05 |
| miR-126 | 5,331 ± | 511 | 2,029 ± | 373 | < 0.01 |
| miR-140-3p | 320 ± | 25 | 567 ± | 22 | < 0.01 |
| miR-148a | 1,680 ± | 278 | 169 ± | 33 | < 0.01 |
| miR-15a | 868 ± | 122 | 143 ± | 35 | < 0.01 |
| miR-195 | 5,193 ± | 568 | 2,267 ± | 712 | < 0.05 |
| miR-202* | 7,125 ± | 735 | 577 ± | 250 | < 0.001 |
| miR-21 | 7,871 ± | 1,345 | 2,284 ± | 943 | < 0.05 |
| miR-23a | 7,247 ± | 265 | 4,436 ± | 317 | < 0.05 |
| miR-23b | 7,622 ± | 177 | 4,290 ± | 317 | < 0.01 |
| miR-24 | 2,625 ± | 225 | 1,691 ± | 219 | < 0.05 |
| miR-27a | 2,040 ± | 138 | 495 ± | 76 | < 0.001 |
| miR-27b | 2,522 ± | 147 | 792 ± | 78 | < 0.001 |
| miR-29a | 5,160 ± | 553 | 1,269 ± | 195 | < 0.01 |
| miR-29b | 561 ± | 91 | 69 ± | 20 | < 0.001 |
| miR-29c | 3,407 ± | 308 | 141 ± | 37 | < 0.001 |
| miR-30a | 914 ± | 82 | 253 ± | 58 | < 0.05 |
| miR-30b | 1,759 ± | 153 | 1,066 ± | 152 | < 0.05 |
| miR-30c | 1,668 ± | 150 | 1,113 ± | 109 | < 0.05 |
| miR-320 | 2,416 ± | 347 | 4,507 ± | 543 | < 0.01 |
| miR-335 | 821 ± | 179 | 136 ± | 34 | < 0.01 |
| miR-34c-3p | 209 ± | 64 | 1,493 ± | 235 | < 0.05 |
| miR-361-5p | 1,347 ± | 139 | 1,944 ± | 88 | < 0.01 |
| miR-365 | 460 ± | 67 | 115 ± | 23 | < 0.01 |
| miR-424 | 824 ± | 163 | 74 ± | 23 | < 0.01 |
| miR-432 | 197 ± | 29 | 693 ± | 131 | < 0.05 |
| miR-451 | 64 ± | 33 | 4,226 ± | 2,809 | < 0.05 |
| miR-574-5p | 244 ± | 86 | 1,919 ± | 470 | < 0.05 |
| miR-768-3p | 771 ± | 280 | 3,699 ± | 753 | < 0.05 |
| miR-768-5p | 857 ± | 353 | 4,863 ± | 1,099 | < 0.05 |
| miR-92a | 1,421 ± | 82 | 2,649 ± | 171 | < 0.001 |
| miR-92b | 428 ± | 32 | 633 ± | 54 | < 0.05 |
| miR-99b | 671 ± | 87 | 1,285 ± | 166 | < 0.05 |

6.4.2 APA miRNAs Targeting the *CYP11B1* and *CYP11B2* Genes.

The role of APA-expressed miRNAs in the regulation of the *CYP11B1* and *CYP11B2* genes was investigated by comparing the miRNA array with the putative target sites identified in these genes by bioinformatic target site (see Chapter 4). APA miRNAs with target sites within these genes are shown in Figure 6-5; 18 miRNAs were predicted to target the 3'UTR of *CYP11B1* and 11 to bind *CYP11B2*. Of these, 8 miRNAs had target sites common to both genes.

Merging the miRNA target site lists for the normal adrenal and APA samples identified that all miRNAs predicted to target the *CYP11B1* or *CYP11B2* 3'UTR are expressed in the normal adrenal gland (Figure 6-6), although some are expressed at significantly different levels between sample groups (Table 6-2). However, 7 miRNAs are only detectable in normal adrenal gland and not in APA samples. Of these, 2 target *CYP11B1*, 1 targets *CYP11B2* and 4 target both genes (Figure 6-6).

The relative, normalised expression levels of the miRNAs expressed in both tissue types at significantly different levels are depicted in Figure 6-7. Two of the *CYP11B1*-targeting miRNAs, miR-23a and miR-23b, are expressed at significantly lower levels in APA tissue than normal adrenal gland, whereas the abundance of miR-432 and miR-768-5p is significantly lower in APA tissue than normal adrenal gland (Figure 6-7 and Table 6-2). Only one miRNA which exclusively targets the *CYP11B2* 3'UTR, miR-125a-5p, is present in different levels being significantly higher in normal adrenal gland than APA tissue (Figure 6-7 and Table 6-2).

Finally, 4 miRNAs predicted to target both *CYP11B1* and *CYP11B2* are differentially expressed in these tissues (Figure 6-7 and Table 6-2). The levels of miR-10b and miR-24 are significantly higher in normal tissue than in APA, whereas miR-140-3p and miR-34c-3p are expressed at higher levels in APA samples relative to normal adrenal tissue (Figure 6-7 and Table 6-2).

It should be reiterated the removal of miR-768-5p from the miRNA repository and therefore does not represent a likely regulator of adrenal physiology.

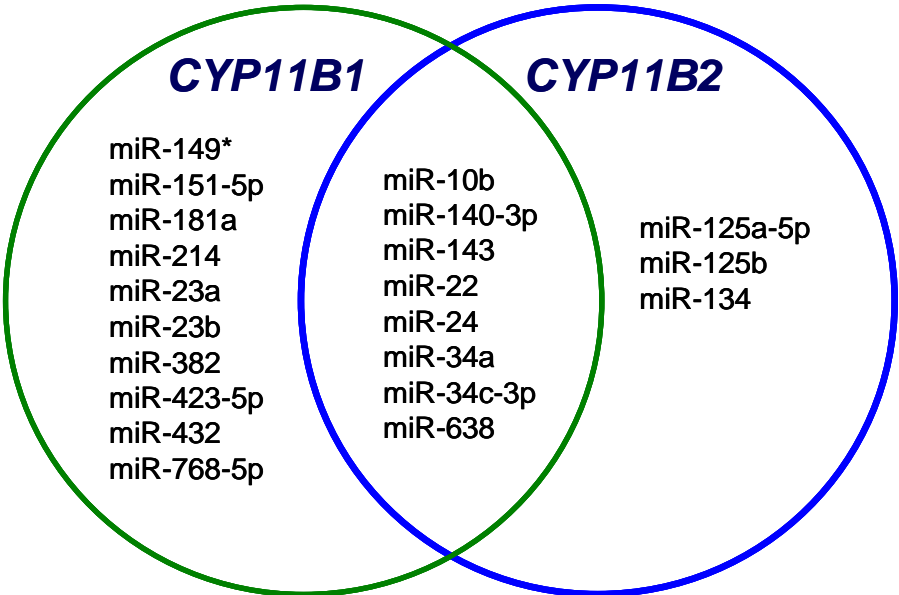


Figure 6-5. Venn Diagram of APA Adrenal miRNAs Predicted to Bind to the *CYP11B1* and *CYP11B2* Genes

Results of miRNA microarray analyses of aldosterone-producing adenoma adrenal tissue combined with data from bioinformatics searches for putative miRNA binding sites in the 3'-UTR of the *CYP11B1* and *CYP11B2* genes.

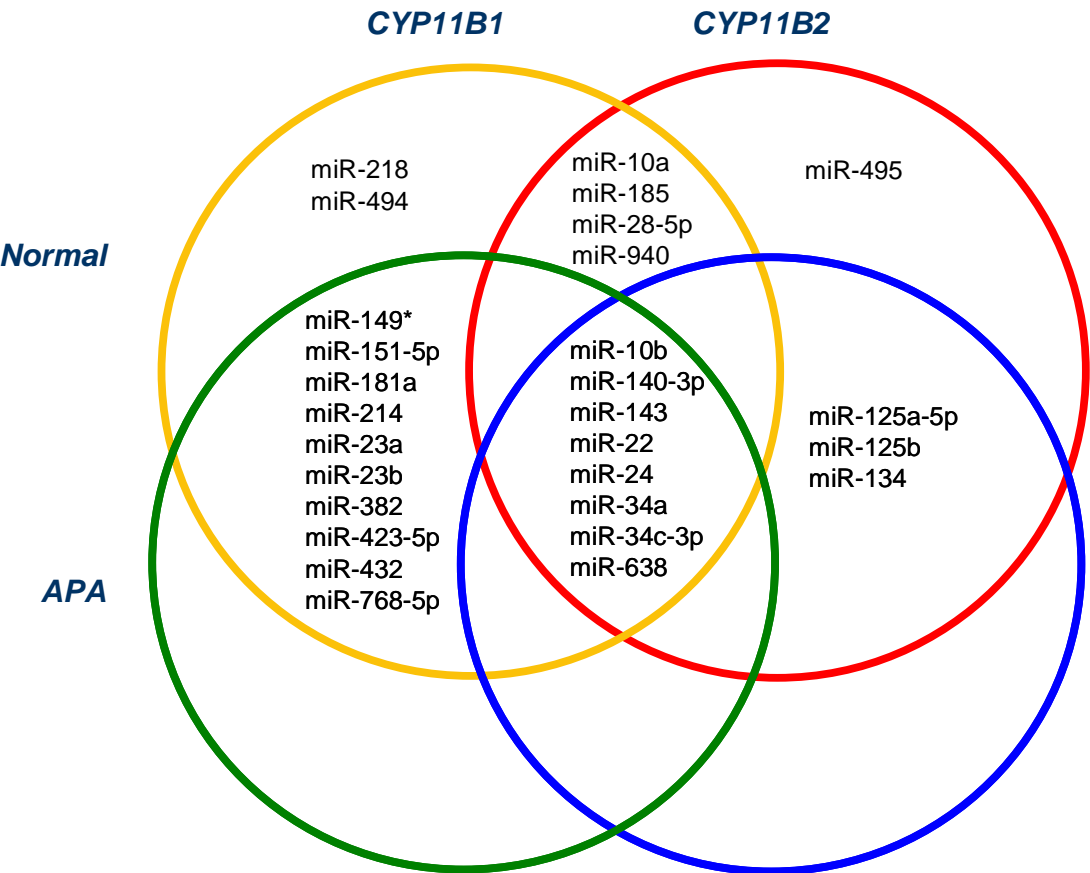


Figure 6-6. Putative miRNA Binding Sites in *CYP11B1* and *CYP11B2* and miRNA Expression in Adrenal Tissue.

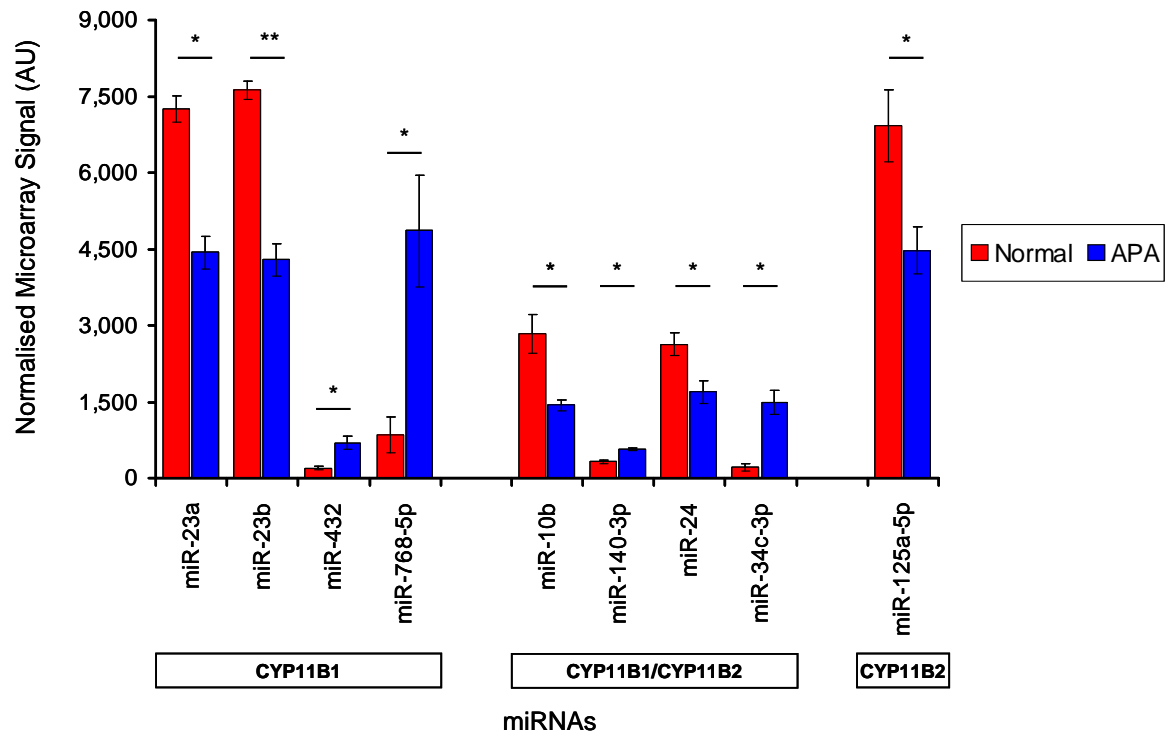


Figure 6-7. Differentially Expressed, Predicted *CYP11B1* or *CYP11B2* Targeting miRNAs.

Results show the relative expression levels of miRNAs in normal adrenal (Red) or APA (blue) tissue. Results are presented according to whether predicted miRNA binding occurs in *CYP11B1* and/or *CYP11B2*. * $p < 0.05$, ** $p < 0.01$.

6.4.3 Genetic Variation at the 3'UTR of *CYP11B1* and *CYP11B2* Genes

Approximately 2,000 base-pairs of the 3'UTR of *CYP11B1* were successfully amplified (Figure 6-8) and sequenced (Figure 6-9) in 26 normotensive patients, which were stratified based on their genotype at the -344 C/T (rs1799998) and intron 2 conversion loci: TT/ConCon (n = 11); CC/WtWt (n = 10); TT/WtWt (n = 5) (Table 6-4). This small sub-set of subjects was chosen to allow the identification of novel SNPs across this region and to ensure all three major haplotypes in the region were represented. Twelve polymorphisms were identified across the region; all were single-base changes (Table 6-3) and the alleles corresponding to each genotype group are shown in Table 6-4. All twelve of the SNPs identified are recorded in the Single Nucleotide Polymorphism Database (dbSNP).

Non-random selection of subjects in this study prevents analysis of allele and haplotypes frequencies or calculation of LD. The pattern of SNPs certainly supports high LD, as is observed across the whole *CYP11B1/B2* locus (Davies *et al.*, 2009). To explore this further, population data from the 1000 genome project was evaluated for each of the SNPs; unfortunately for two SNPs (rs5301 and rs72552270) no population data was available (Table 6-5). Analysis indicates that for the majority of SNPs the two alleles are present at approximately equal frequency in a Caucasian population, but for three SNPs (rs61752812, rs1752809, rs61752805) the minor allele is only rarely observed in a Caucasian population; therefore were excluded from linkage disequilibrium analysis. The LD plot demonstrated a high degree of Linkage Disequilibrium between seven 3'UTR SNPs (Figure 6-10). Comparable population data was not available for *CYP11B1* promoter SNPs (rs4471016 and rs4313136) thus, the level of linkage disequilibrium across the gene could not be determined.

The 3'UTR of *CYP11B2* has previously been sequenced in the same cohort of patients by other members of the group during a full gene SNP discovery investigation (Barr *et al.*, 2007). This analysis identified four genetic variants within the 3'UTR, the details of the variants - 3 single-base changes and a 3-base insertion (rs113094040) - are described in Table 6-6. The base changes

attributed to the polymorphisms and their association with the -344 C/T and intron 2 polymorphisms are shown in Table 6-7. One of these polymorphisms appears to be a novel finding; no previous report of it was found in dbSNP, and as such does not have a reference sequence (rs) number. Again, for statistical analysis genotype from genotype data taken from a small subset (low coverage panel) of subjects in the 1000 genome project (Table 6-8); this could only be achieved for two SNPs (rs28491316 and rs3802230) and *CYP11B2* -344C/T promoter SNP (rs1799998). All three of these variants are in high LD (Figure 6-12).

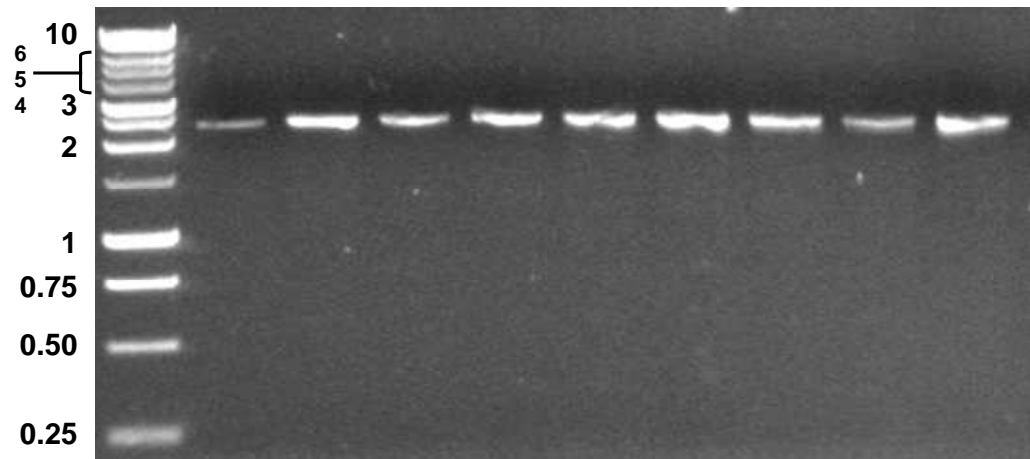


Figure 6-8. *CYP11B1* 3'UTR PCR Amplification in MONICA Patients.

PCR product from a random selection of MONICA patients was resolved on a 0.7% agarose gel. Promega 1 kb ladder was used for size determination; sizes indicated are in kilobase pairs.

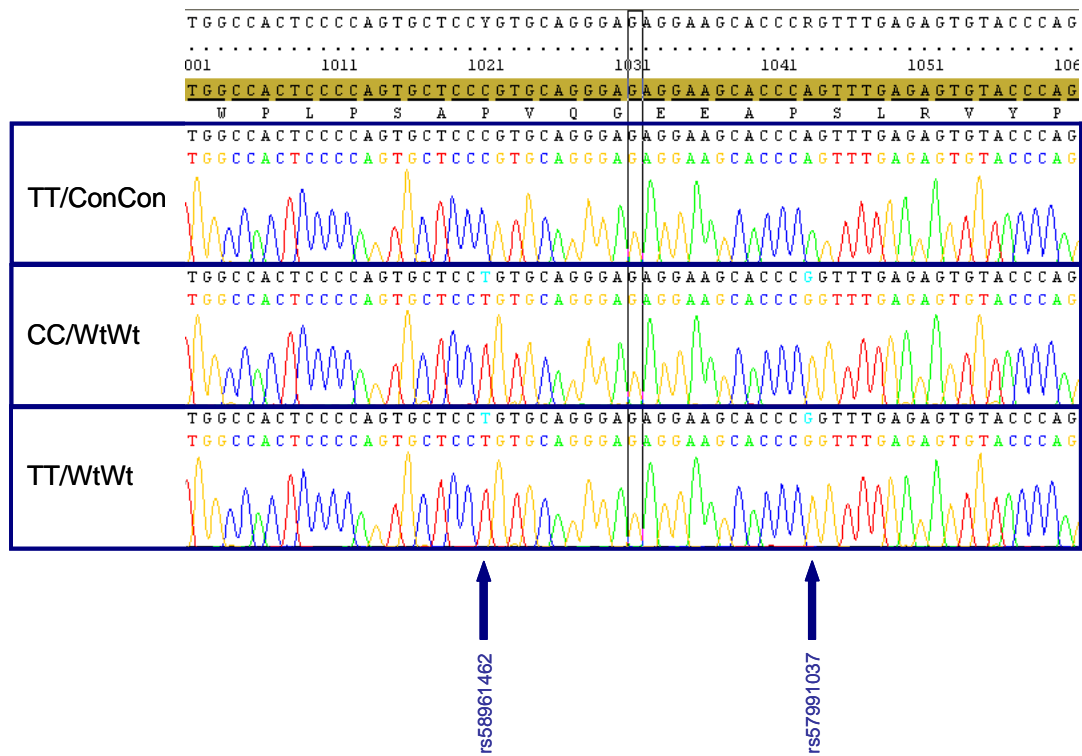


Figure 6-9. Genotype Analysis of *CYP11B1* 3'UTR

An example of the electropherogram reads from one subject of each haplotype group (indicated on the left hand-side; -344(promoter SNP)/intron conversion status). This example shows the C/T SNP (re58961462) and the A/G SNP (rs57991037).

Table 6-3. Characteristics of Polymorphisms Genotyped in the *CYP11B1* 3'UTR.

| SNP | Postion | Location | Alleles |
|------------|-----------|----------|---------|
| rs4736312 | 143953937 | 7926 | T /G |
| rs1134096 | 143954223 | 7640 | G /T |
| rs1134095 | 143954290 | 7573 | C /T |
| rs61752812 | 143954372 | 7491 | G /A |
| rs61752809 | 143954501 | 7362 | A /G |
| rs7003319 | 143954747 | 7116 | A /G |
| rs5017238 | 143954769 | 7094 | C /T |
| rs61752805 | 143954866 | 6997 | G /C |
| rs12543598 | 143955318 | 6545 | A /C |
| rs5299 | 143955471 | 6392 | A /G |
| rs5301 | 143955669 | 6590 | A /G |
| rs72552270 | 143955891 | 6812 | G /A |

Table 6-4. Polymorphisms Identified in the *CYP11B1* 3'UTR (grouped according to -344 and IC genotype).

| | Position | | | | | | | | | | | |
|-----------|----------|------|------------------|------|------------------|------|------|------------------|------------------|------|------|------|
| | 6392 | 6545 | 6590 | 6812 | 6997 | 7094 | 7116 | 7362 | 7491 | 7573 | 7640 | 7926 |
| TT/ConCon | A | A | A | G | G | C | A | A | G | C | G | T |
| CC/WtWt | G | C | G | A | G/C [#] | T | G | A | G | T | T | G |
| TT/WtWt | G | C | G/A [*] | A | G | T | G | G/A [*] | G/A [*] | T | T | G |

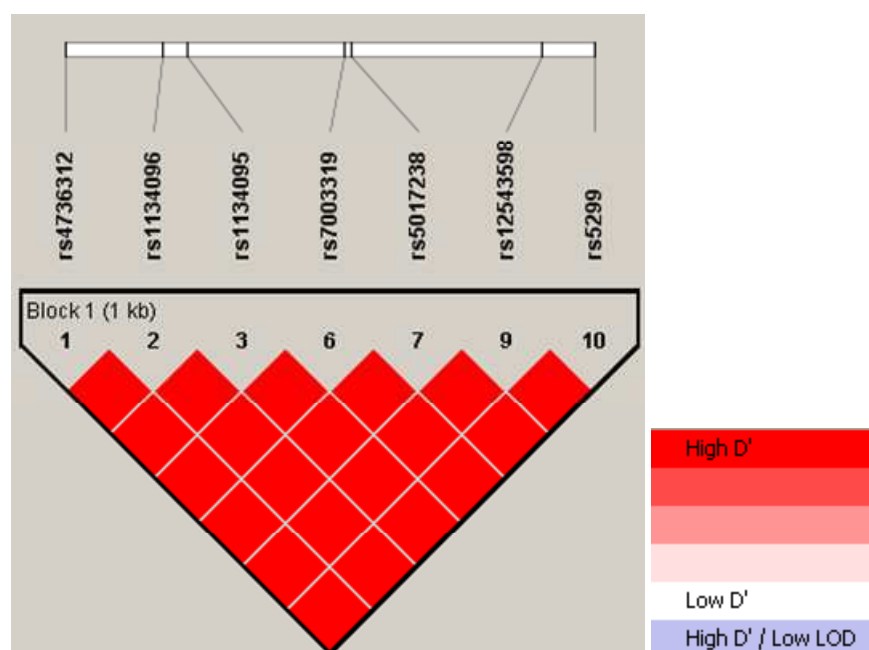
[#]: 2 subjects were heterozygous (G/C) at position 6997, 8 subjects were homozygous (G/G)

^{*}: 3 subjects were heterozygous (G/A) at positions 6590, 7362 and 7491. One subject was homozygous (G/G) and one homozygous (A).

Table 6-5. Statistical Analysis of *CYP11B1* Polymorphisms.

| SNP | Postion | | MAF | Obs. Het | HWE (p) |
|------------|-----------|----------|------------------------------|----------|---------|
| rs4736312 | 143953937 | 3'UTR | 0.48 | 0.50 | 1.00 |
| rs1134096 | 143954223 | 3'UTR | 0.49 | 0.48 | 0.95 |
| rs1134095 | 143954290 | 3'UTR | 0.49 | 0.48 | 0.95 |
| rs61752812 | 143954372 | 3'UTR | 0.06 | 0.12 | 1.00 |
| rs61752809 | 143954501 | 3'UTR | 0.06 | 0.12 | 1.00 |
| rs7003319 | 143954747 | 3'UTR | 0.49 | 0.48 | 0.95 |
| rs5017238 | 143954769 | 3'UTR | 0.49 | 0.48 | 0.95 |
| rs61752805 | 143954866 | 3'UTR | 0.04 | 0.08 | 1.00 |
| rs12543598 | 143955318 | 3'UTR | 0.48 | 0.45 | 0.57 |
| rs5299 | 143955471 | 3'UTR | 0.49 | 0.48 | 0.95 |
| rs5301 | 143955669 | 3'UTR | No population data available | | |
| rs72552270 | 143955891 | 3'UTR | No population data available | | |
| rs4471016 | 143963087 | Promoter | No population data available | | |
| rs4313136 | 143963117 | Promoter | No population data available | | |

Data taken from Ensembl release 66 (Feb 2012) using genotype data from the 1000 genome CEU population, low coverage panel.

**Figure 6-10. LD Plot and Haplotypes of Polymorphisms in the *CYP11B1* 3'UTR.**

The LD plot (generated with the D' method) of *CYP11B1* polymorphisms, generated using genotype data from the 1000 genome CEU population, low coverage panel.

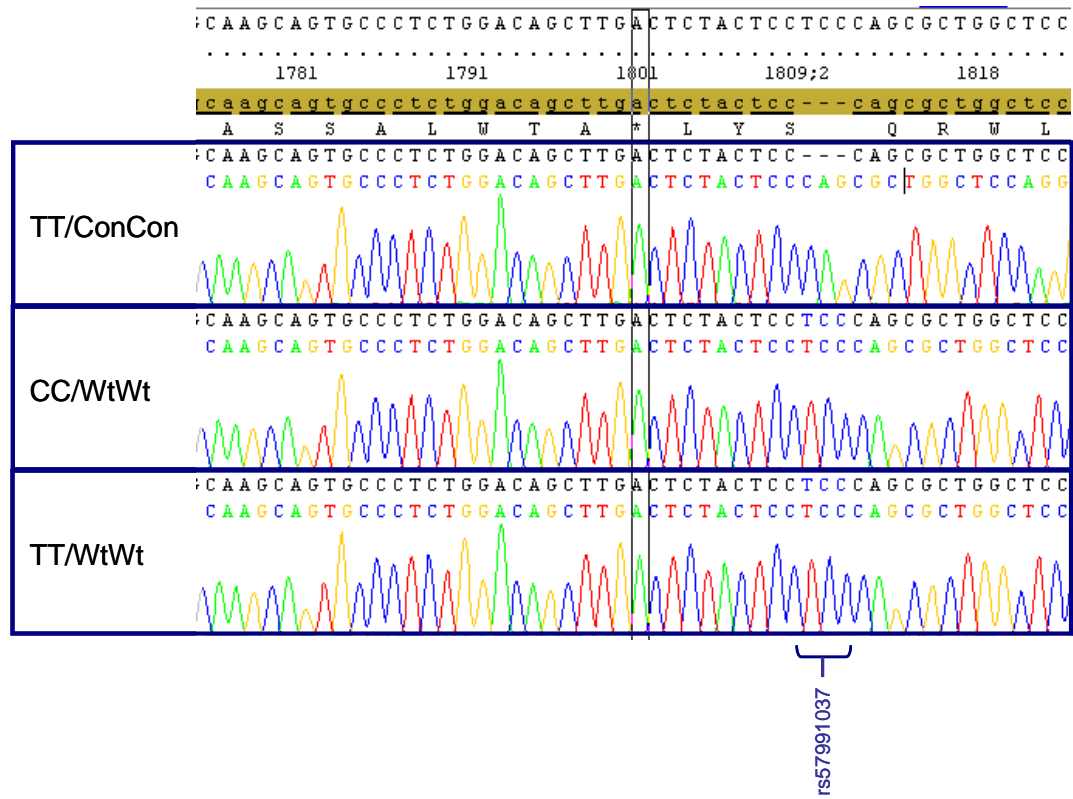


Figure 6-11. Genotype Analysis of *CYP11B2* 3'UTR

An example of the electropherogram read from one subject of each genotype group (indicated on the left hand-side). This example shows the TCC insertion (rs57991037).

Table 6-6. Characteristics of the Polymorphisms Genotyped in the *CYP11B2* 3'UTR.

| SNP | Postion | Location | Alleles |
|-------------|-----------|----------|---------|
| rs113094040 | 143993104 | 6756 | - /TCC |
| rs28491316 | 143992661 | 7199 | G /A |
| rs3802230 | 143992864 | 6996 | G /T |
| Unassigned | 143992411 | 6543 | A /G |

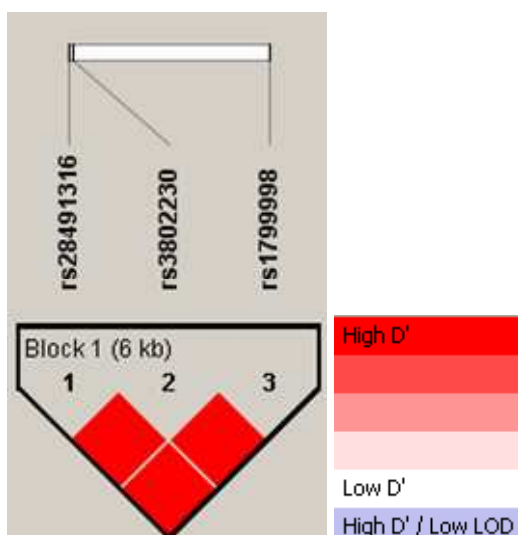
Table 6-7. Polymorphisms Identified in the *CYP11B2* 3'UTR (grouped according to -344 and IC genotype).

| | Position | | | |
|-----------|----------|------|------|------|
| | 6543 | 6756 | 6996 | 7199 |
| TT/ConCon | A | --- | G | G |
| CC/WtWt | G | TCC | T | A |
| TT/WtWt | G | TCC | T | G |

Table 6-8. Statistical Analysis of *CYP11B2* Polymorphisms.

| SNP | Postion | | MAF | Obs. Het | HWE (p) |
|-------------|-----------|----------|------------------------------|----------|---------|
| rs1799998 | 143999600 | Promoter | 0.43 | 0.50 | 1.00 |
| rs28491316 | 143992661 | 3'UTR | 0.45 | 0.47 | 0.8 |
| rs3802230 | 143992864 | 3'UTR | 0.50 | 0.50 | 1.00 |
| Unassigned | 143992411 | 3'UTR | No population data available | | |
| rs113094040 | 143963117 | 3'UTR | No population data available | | |

Data taken from Ensembl release 66 (Feb 2012) using genotype data from the 1000 genome CEU population, low coverage panel.

**Figure 6-12. LD Plot and Haplotypes of Polymorphisms in the *CYP11B2* 3'UTR.**

The LD plot (generated with the D' method) of *CYP11B2* polymorphisms, generated using genotype data from the 1000 genome CEU population, low coverage panel.

6.4.4 Impact of Polymorphisms on miRNA Binding Sites

The putative binding sites of miRNAs expressed in the normal adrenal gland (Figure 4-8) were mapped to the 3'UTR of the *CYP11B1* or *CYP11B2* gene to determine whether genetic variation in this region could disrupt a miRNA binding site (Figure 6-13 and Figure 6-14).

Three *CYP11B1* 3'UTR SNPs lie in putative miRNA binding sites: rs5299 and miR-140-3p; rs57250946 and miR-22; rs61752812 and miR-494 (shown in red in Figure 6-13). According to TargetScan or microrna.org predictions, none of the SNPs are located in the seed site of the miRNA binding site, or in other complementary base-matches between the 3'UTR and the other regions of the miRNA. Furthermore, 13 miRNA target-sites lie within 50 base-pairs of a SNP (indicated in yellow in Figure 6-13) whereas, the binding sites for the other 10 miRNAs do not lie in close proximity to a 3'UTR SNP.

Of the four polymorphisms in the *CYP11B2* 3'UTR (Figure 6-14), only one SNP is located in a miRNA binding site: rs380223 which lies in the common miR-10a/miR-10b region; again this SNP does not lie in the seed region. Another 9 miRNA binding sites lie within 50 bases of a SNP (yellow in Figure 6-14) and 6 binding sites were not in close proximity to a polymorphism.

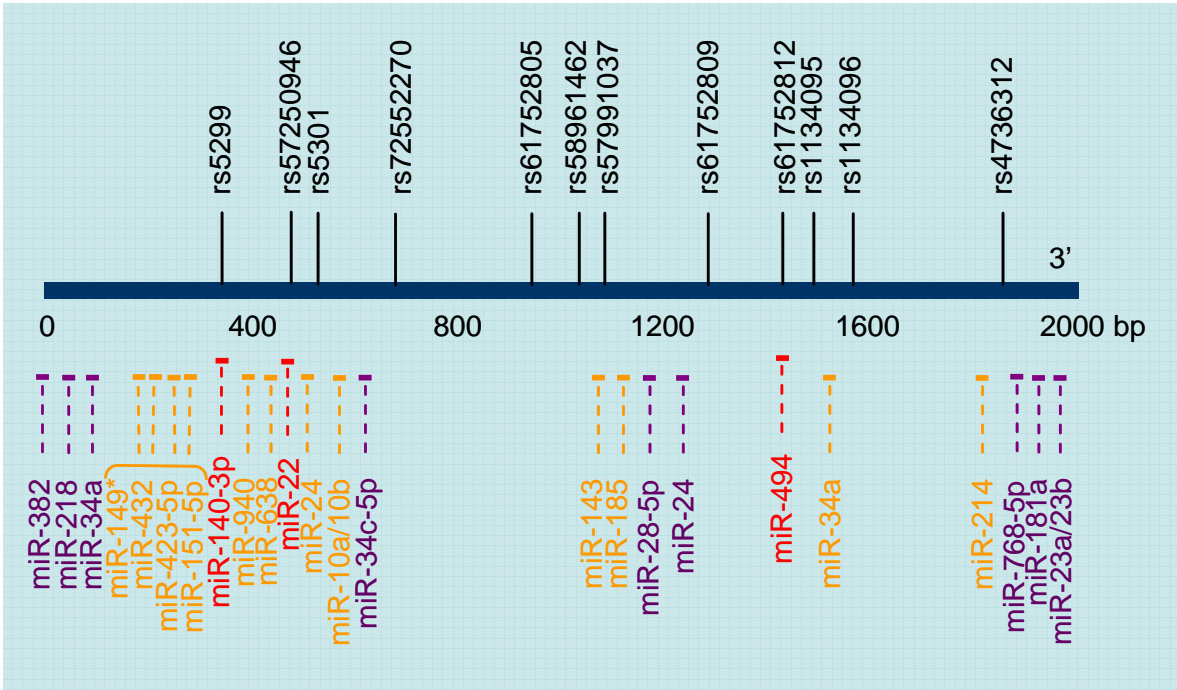


Figure 6-13. Schematic Diagram of SNPs and miRNA Binding Sites in the *CYP11B1* 3'UTR.

Location of single nucleotide polymorphisms (SNPs) on the 3'UTR of the *CYP11B1* gene. Adrenal-expressed miRNA binding sites are colour-coded. Red: SNP located in binding region; Yellow: SNP with 50 base-pairs (bp) of binding site; Purple: binding site not in close proximity to SNP. Diagram not to scale.

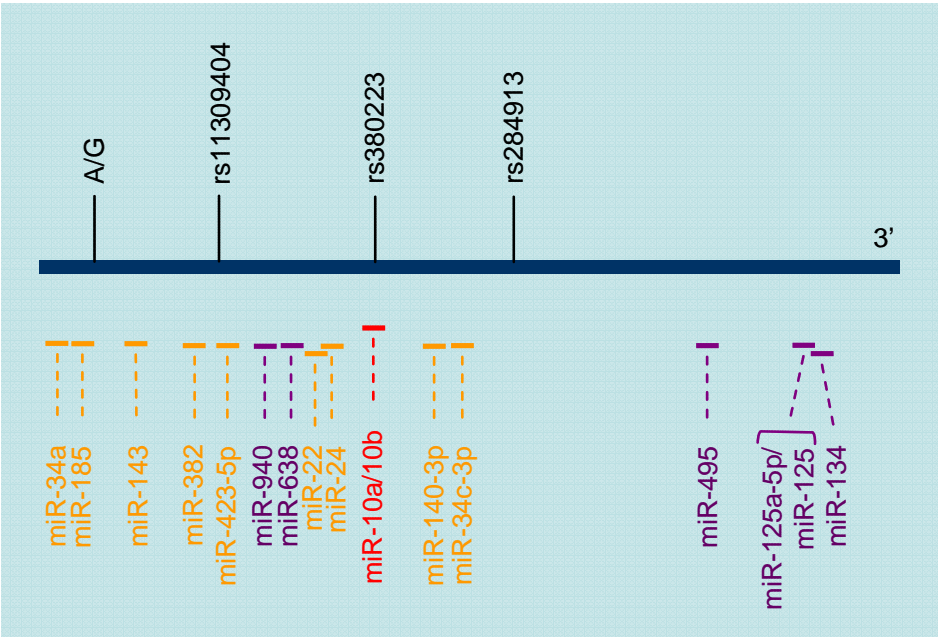


Figure 6-14. Schematic Diagram of SNPs and miRNA Binding Sites in the *CYP11B2* 3'UTR.

Location of single nucleotide polymorphisms (SNPs) on the 3'UTR of the *CYP11B2* gene. Adrenal-expressed miRNA binding sites are colour-coded. Red: SNP located in binding region; Yellow: SNP with 50 base-pairs (bp) of binding site; Purple: binding site not in close proximity to SNP. Diagram not to scale.

6.5 Discussion

The experiments presented in this chapter investigated the role of miRNA in adrenal pathologies and the influence of SNPs in the 3'UTR of the *CYP11B1* and *CYP11B2* genes on miRNA binding.

This study successfully determined the miRNA expression profile of APA samples, identifying several miRNAs not expressed in normal tissue and many more with differential expression levels. However, the observed differences in miRNA expression shown and discussed here need to be validated by qRT-PCR analysis in order to confirm the accuracy of the array data and to determine more precisely the magnitude of these changes. This work will be conducted in the future, but time constraints prevent their presentation in this thesis.

The APA tissue samples were fixed in formalin and embedded in paraffin blocks (FFPE), which is normally problematic as it leads to degradation of longer RNA species. However, due to their small size, miRNAs can successfully be isolated and measured in archived preserved tissue (Xi *et al.*, 2007; Li *et al.*, 2007). As shown in this study, utilising archived tissue increases the range of viable tissues available for miRNA studies. Four samples were used in this study to establish the APA miRNA expression profile and this number is comparable to those used in the two previous adrenal miRNA investigations (Soon *et al.*, 2009; Schmitz *et al.*, 2011). Given the appropriate sample number for human adrenal miRNA profiling and the low intrasample variation in expression pattern, the results appear to confirm the homogeneity of the selected samples and the accuracy of expression measurements.

Despite the miRNA microarrays for the two sample types (APA and normal adrenal) being performed at different times, care was taken to permit data-sets to be compared accurately; arrays were performed using the same method, at the same supplier and using the same microfluidic chips with identical miRNA coverage. Post-array analysis utilised internal spike-in controls to cross-normalise the array data output, allowing signals to be compared between chips. There was a good number of APA-expressed miRNAs with target sites in the *CYP11B1* and/or *CYP11B2* genes and this was similar to those previously observed

for normal adrenal tissue (Chapter 4). Furthermore, there was overlap of miRNA species with several being expressed in both sample types. This adds confidence to the miRNA expression profile data generated this part of the study and indicates that the miRNA coverage and tissues types were appropriate and of suitable stringency.

Seven miRNAs which were predicted to have binding sites in *CYP11B1* and *CYP11B2* genes were only expressed in normal adrenal gland. Additionally, nine miRNAs targeting both *CYP11B1* and *CYP11B2* were expressed at significantly different levels in the two tissue types. This may suggest that these miRNAs are important in regulatory processes surrounding adrenal pathology. Moreover, several of the miRNAs identified have also been implicated in tumorigenesis: miR-24 represses the translation of p16 (Cyclin-dependent kinase inhibitor 2A) (Lal *et al.*, 2008) and miR-10b is strongly implicated in breast cancer (Ma *et al.*, 2007). This may indicate that, in addition to regulating adrenal-specific genes, these miRNAs can change cell cycle regulation or cell morphology, leading to neoplastic growths.

Two of the miRNAs which target *CYP11B1* and *CYP11B2*, miR-23 and miR-24, are transcribed in two separate clusters (Section 6.5). All cluster members (including miR-27a and miR-27b which are not predicted to bind *CYP11B1* or *CYP11B2*) are expressed at lower levels in APA samples. The miRNAs from the miR-24-2 cluster are located on a intergenic region, whereas the genomic locus which includes the miR-24-1 cluster, *C9orf3*, is believed to encode a novel metalloprotease called aminopeptidase-O (AP-O) (Diaz-Perales *et al.*, 2005). *AP-O* may be a novel dysregulated gene in APA. An attempt was made to measure the relative mRNA levels of *AP-O* in normal and APA samples but the RNA quality of the samples was not suitable for mRNA qRT-PCR analysis. The role of AP-O in adrenal physiology has not been studied, but AP-O is proposed to be important in vascular cell biology (Xi *et al.*, 2007) and another aminopeptidase (AP-A) is integral to the RAAS, cleaving angiotensin II into angiotensin III; it also plays a critical role in blood pressure regulation (Ahmad and Ward, 1990; Mitsui *et al.*, 2003). Further investigation of the AP-O protein and the miRNAs transcribed from its eight introns is required in order to establish their role in adrenal function.

This study also analysed 26 subjects who had been selected from a larger normotensive cardiovascular population study on the basis of their genotype at the -344 C/T and IC loci. These polymorphisms have been shown to be linked to increased aldosterone production, inefficient 11 β -hydroxylation and hypertension (Davies *et al.*, 2009). They are also in tight LD, the frequencies of their haplotypes in a normotensive Caucasian population are: CC/WtWt (45%), TT/ConCon (38%) and T/Wt (16%) (Davies *et al.*, 1999).

Previous sequencing analysis identified 4 polymorphisms in the *CYP11B2* 3'UTR and this current investigation identified 12 further SNPs in the *CYP11B1* 3'UTR. Population data from the 1000 genome project found that seven *CYP11B1* 3'UTR SNPs are in high LD and additionally, two *CYP11B2* 3'UTR and the -344 promoter are in high LD. This agrees with previous assessment of LD across the locus (Barr *et al.*, 2007). Therefore, the causative mechanism governing the association of the -344C/T SNP with hypertension (Sookoian *et al.*, 2007) or the *CYP11B1* promoter SNPs with inefficient 11 β -hydroxylation (Barr *et al.*, 2006) could lie with variation in the 3'UTR.

Currently, the molecular mechanism to explain the relationship between *CYP11B1/CYP11B2* genotype and blood pressure phenotype remains elusive. A study of genetic variants and gene expression in brain cortical samples showed that genes with a high degree of variation are more likely to be regulated by miRNAs than less variable genes (Zhang and Su, 2008). Therefore, regulation of the *CYP11B1* and *CYP11B2* genes by miRNA offers a novel method of gene regulation, which may control basal mRNA expression levels but may also be influential in cases of altered gene transcription and steroid production. As the subjects in this study were drawn solely from a normal Caucasian normotensive population (Fraser *et al.*, 1999), the possibility exist that, in a hypertensive population, the frequencies of these haplotypes may vary, or that there may even be additional novel SNPs. Additionally, this study only evaluated 26 subjects which would not detect very rare SNPs *i.e.* those present in less than 0.01% of the population.

This study confirmed that the highly polymorphic nature of this locus extends to the 3' regulatory region, but none of the 3'UTR SNPs were located in the seed region of a putative miRNA target site. The seed site only accounts for 8 bases of

a miRNA and complementary binding in other regions of the miRNA have been shown to be important (Grimson *et al.*, 2007; Shin *et al.*, 2010) but, according to my bioinformatic analysis, no SNP is located in a complementary mRNA:miRNA base-pairing region either. Yet, SNPs may still influence miRNA action in other ways; mismatches and bulges are frequently tolerated by miRNAs, as are G:U wobble. Therefore, it is possible that a SNP in a miRNA-binding region may alter the binding capacity and/or structure of miRNA:target interactions. Thus, while the influence of SNPs may extend beyond simple base-pairing, this would need to be tested *in vitro*. Furthermore, the collective influence of SNPs with a haplotype may be more important than individual SNPs, and be more representative of actual physiology. One way of testing this would be to mutate the 3'UTR of the pEZX reporter construct (Chapter 3), in order to recreate the various haplotypes then test the relative responses *in vitro*. Sequence analysis of the pEZX-B1 and pEZX-B2 plasmid constructs (Section 3.4.3) indicates that they contain the alleles identified in the CC/WtWt subjects (Table 6-4 and Table 6-7). Furthermore, experiments presented in this thesis have tested miRNA action in the H295R cell line; the 3'UTR of *CYP11B1* or *CYP11B2* not been sequenced in DNA isolated from these cells. They have however, been sequenced for the common polymorphism and contain the CC allele at -344 and the 'wild type' sequence at intron 2. Based on information in this chapter the sequences at the 3'UTR could be inferred, but direct sequencing would need to be carried out to confirm this.

Bioinformatic analysis in Chapter 4 confirmed that no pri-miRNA or pre-miRNA sequences are located in the genomic region encompassing these genes: ruling out the possibility of an intronic SNP that causes altered miRNA-expression or function. Investigating the other possible *cis*-factors are beyond the scope of this project but, should a *CYP11B1* or *CYP11B2*-regulating miRNA(s) be identified, then exploration of the epigenetic regulation of that miRNA would clearly prove to be of interest (Lehmann *et al.*, 2008; Ryan *et al.*, 2010).

Finally, polymorphic variations need not be located in a miRNA-binding site to influence miRNA-action; there is evidence that 3'UTR SNPs located adjacent to miRNA binding sites have a profound influence on miRNA-mediated regulation. The C829T polymorphism in the 3'UTR of the human *DHFR* (Dihydrofolate reductase) gene is located 14 base-pairs downstream of a verified miR-24 target

site. Cells expressing the T allele (at position 829) DHFR exhibit no repression when miR-24 levels are increased, in contrast with the observed effect in wild-type (C allele at position 829) DHFR cells (Mishra *et al.*, 2007). It is possible that SNPs in the *CYP11B1* and *CYP11B2* genes are acting in a similar manner. Such SNPs could influence miRNA regulation by altering the secondary structure of the 3'UTR, which may in turn affect the miRNA target-site accessibility or prevent the association of the large protein complexes that make up RISC (RNA-induced silencing complex) and which are crucial to miRNA action (Kertesz *et al.*, 2007; Mishra *et al.*, 2007; Jinek and Doudna, 2009).

In summary, miRNA expression levels were shown to be altered in APA samples relative to normal controls, and this could be indicative of their role in the pathogenesis of adrenal disease and in hypertension. Additionally, some miRNA binding sites in the *CYP11B1* and *CYP11B2* genes are located in regions which possess a common genetic variant, while others are located proximal to variants and may also play a role in miRNA regulation. Further *in vitro* studies would be helpful in understanding the influence of both individual SNPs and the full haplotype on miRNA regulation.

7 General Discussion

Essential hypertension (EH) is a common condition and a risk factor for cardiovascular and cerebrovascular disease. The aetiology of EH is unknown but both lifestyle and genetic factors are known to contribute. The genes involved in the adrenal corticosteroid biosynthesis pathway are a major candidate system; understanding the regulation of these genes should increase our current understanding of EH.

There are multiple factors which control the regulation of aldosterone and cortisol production in the adrenal cortex, including those which alter the expression of the *CYP11B2* and *CYP11B1* enzymes that respectively catalyse the final steps in their production. The roles these enzymes (and their genes) play in the regulation of blood pressure, and their contribution to the development of hypertension, were outlined in [Chapter 1](#). The aim of this project was to investigate the existence of a novel *CYP11B1* and *CYP11B2* regulating mechanism involving miRNAs.

This study is the first to examine, directly, the regulation of *CYP11B1* and *CYP11B2* by miRNAs, and this was accomplished using both direct and indirect methods performed *in vitro*, *in silico* or *ex vivo*. In [Chapter 3](#), the levels of mRNA encoding Dicer mRNA (an important enzyme in the process of miRNA maturation) was reduced in H295R cells. Knockdown of miRNA levels by this method increased the abundance of several key corticosteroidogenic mRNAs (including *CYP11B1* and *CYP11B2*) and of their corresponding steroid products. Cloning of the *CYP11B1* or *CYP11B2* 3'UTR into a reporter construct indicated that these regions were capable of, and sufficient to, exert a negative regulatory effect on the expression of a reporter gene. It was further demonstrated that this regulation is responsive to AngII stimulation. Taken together, these studies supported the initial hypothesis of miRNA-mediated regulation of adrenal corticosteroidogenesis.

The studies in [Chapter 4](#) utilised bioinformatic prediction algorithms to identify miRNAs likely to target the 3'UTR of *CYP11B1* and *CYP11B2* mRNAs. Based on miRNA target site prediction and analyses of the 3'UTR sequences (which included relative length, predicted sequence conservation and RNA secondary structure), *in silico* methods concluded that it was possible that miRNAs can target *CYP11B1* and/or *CYP11B2* in this way. Prediction of biological processes by

bioinformatic algorithms is generally problematic; the imperfect nature of miRNA binding to the target site complicates it further. The currently available databases are built on our best understanding of miRNA biology, but the novelty and rapid development of the field entails continual modification of their algorithms. Even in the three-year duration of this study, predicted target sites have changed; interestingly my validation studies ([Chapter 5](#)) support the more recent predictions suggesting that these updates have, on the whole, tended towards improvements. There will always be an inherent error associated with bioinformatic predictions and this study has identified several false-positive predictions; one would imagine that the frequency of these will be reduced as algorithms are refined. However, this study has not investigated the presence of false-negative target site predictions. Recent findings have identified miRNA-mediated regulation of mRNAs that do not possess strict base-pairing complementarity in the seed region (Grimson *et al.*, 2007; Shin *et al.*, 2010). This would, at least, suggest the possibility of false-negative predictions given that many of the databases are designed to favour seed site sequence matches. To exclude this possibility, the binding site properties of all adrenal miRNAs would need to be tested *in vitro* with *CYP11B1* and *CYP11B2* 3'UTR requiring a lot of time and expense. Therefore, the rationale in this study was to use five databases in order to broaden the prediction and include algorithms of varying stringencies. As these algorithms continue to develop the list of potential targeting miRNAs will need to be updated and tested, as appropriate.

miRNA expression analysis was performed in [Chapter 4](#) for normal human adrenal gland samples and in [Chapter 6](#) for aldosterone-producing adenoma samples. miRNA microarray and qRT-PCR experiments both identified the presence of specific miRNAs within these tissues; in total, 103 miRNAs were detected above a pre-determined threshold in the normal adrenal gland, compared with 67 in the APA samples. A microarray signal intensity of 500 arbitrary units (AU) was chosen, based on advice from the microarray chip manufacturer that presumably takes into account the scanner's lower limit of detection and the efficiency of hybridisation. Of course several miRNAs expressed marginally below this borderline may still be of importance in adrenal physiology. One obvious reason for this is that a miRNA may only be expressed in a certain adrenal cell type, meaning its concentration is diluted within the heterogeneous adrenal cell

populations analysed here. Zonal micro-dissection and subsequent expression analysis (presumable by qRT-PCR, as the quantity of isolated RNA would be low) would help to determine if this is the case. Analysis of the microarray data requires a cut-off value to be selected and enforced in order to avoid both experimental noise and error; the threshold of 500 AU used here enabled the identification of a manageable number of adrenal miRNAs to be carried forward for subsequent investigation. It was also logical to focus initial studies on miRNAs expressed at higher levels given their likelihood of producing more pronounced biological response.

The studies described in Chapter 5 involved the *in vitro* overexpression or competitive inhibition of miRNAs in order to assess the validity of miRNA binding sites predicted in the previous chapters. The effect of altered miRNA levels was assessed by reporter construct assays and by direct measurement of mRNA and steroids. These studies were useful for screening the predicted miRNA target sites; several of these were shown to be false positives, while a small number were found to be ambiguous and one showed an action opposite to that generally accepted to be exerted by miRNAs; others were successfully validated. The miRNA that gave the most striking and consistent results for targeting both *CYP11B1* and *CYP11B2* was miR-24. Analysis of adrenal miRNAs predicted only to target the *CYP11B2* 3'UTR confirmed miR-125a-5p and miR-125b as novel regulators, although effects on steroid secretion are still to be assessed.

Expression levels of miR-24 (and of a number of other miRNAs) were shown to be dysregulated in APA samples relative to normal adrenals. This raises the possibility of a role for miR-24 in adrenal pathology and this requires further investigation, possibly utilising *in vivo* studies, for example altering the levels of miR-24 in rats and measuring their steroid production, level of *CYP11B1* and *CYP11B2* expression and cardiovascular parameters.

The experimental work described in this thesis has identified the importance of miRNA-mediated regulation to the adrenal gland. There are several interesting further experiments which now suggest themselves. Firstly, examining the spatial expression of individual miRNAs in adrenal sections would be of interest; there are a number of methods through which this can be achieved, such as by *in situ* hybridisation using either fluorescent probes (Silahtaroglu *et al.*, 2007) or

labelled LNA probes (Nelson *et al.*, 2006). These have been successfully used to identify miRNA localisation in formalin-fixed, paraffin-embedded preserved tissue samples (Nelson *et al.*, 2006) and this is especially pertinent given the difficulty in obtaining good-quality fresh or frozen human adrenal gland sections. Assessment of adrenal miRNA localisation would determine whether a particular miRNA is universally expressed, is confined to a specific zone or exhibits differential expression levels across cell types. The zone-specific expression of *CYP11B1* and *CYP11B2* means that information gained from these experiments could clarify miRNAs' relative impact on these genes.

Secondly, to establish whether miRNAs identified in the present study contribute to other adrenal pathologies. The information gained by doing so may help to further narrow down those miRNAs that target *CYP11B2*. To investigate miRNA involvement in essential hypertension (EH), it would be desirable to examine miRNA expression in the adrenal glands of EH subjects (specifically those with a high ARR), but practical and ethical considerations make this impossible. Where human studies are impractical, it is usual to turn to animal models, but much is still not known about the similarity and conservation of miRNA-mediated mechanisms across species. While, superficially, it may seem attractive to examine the impact of miR-24 knockout on steroid secretion and blood pressure in a mouse model, many differences exist that could render its relevance to human negligible. These include important physiological differences (corticosterone is the major rodent glucocorticoid), before one even considered whether miRNA target sites or expression patterns are maintained across species. Although this may prove to be the case, careful and extensive studies would have to be conducted before the suitability of such animal models was confirmed.

However, human studies may not be reliant on prohibitively invasive methods in order to analyse miRNA action recent publications have successfully demonstrated that miRNAs levels can be measured in blood (Section 1.8.2). That is miRNAs expressed in one organ can be released into the circulation and travel to act in a different organ, therefore miRNAs may act like a novel endocrine signal. In time, develop into a novel diagnostic tool or permit the generation of miRNA expression profiles indicative of the EH patient.

This study has only investigated the role of individual miRNAs in gene regulation but, in normal physiology, it is likely that multiple miRNAs act to regulate the abundance of mRNAs and that the regulation of the miRNAs themselves is crucial to their effect. Furthermore, post-transcriptional regulation by miRNAs may only constitute to a small proportion of the total regulatory influences exerted over the *CYP11B1* and *CYP11B2* genes. Other known or potential factors that contribute to their regulation include epigenetic modulation (e.g. methylation or histone acetylation), transcription factors and post-translational mechanisms (e.g. phosphorylation). In addition to the regulation of *CYP11B1* and *CYP11B2*, a greater knowledge miRNA-mediated regulation throughout adrenal gland may contribute to a better understanding of the regulatory pathways that control adrenal development (Huang and Yao, 2010), adrenal cell differentiation and medullary catecholamine synthesis.

In the more distant future, the regulatory pathways identified in this thesis may prove useful as novel diagnostic tools (possibly through stratification of patient groups) and therapies. Currently, there are no specific aldosterone synthase inhibitors on the market; results presented in this thesis show potential for the development and use of miRNA-based therapies to treat disorders of aldosterone production, particularly hypertension. As previously mentioned there are already early-stage clinical trials of miRNA-targeted treatments in progress (Section 1.8.1), and, as the field continues to mature, the range of conditions for which miRNAs are used may become many and diverse.

In summary, the main findings of this thesis include: the identification of the a general mechanism of miRNA-mediated regulation in an adrenal cell line; the determination of miRNA expression profiles for normal adrenal gland and APA samples; the identification of predicted miRNA binding sites in the 3'UTR of *CYP11B1* and *CYP11B2*, and the development of *in vitro* systems to test and validate several of these. The most prominent regulatory mechanism identified in the course of this project involved the direct and canonical regulation of *CYP11B1* and *CYP11B2* mRNA abundance and steroid secretion by miR-24. Additionally, levels of miR-24 were found to be lower in APA samples than in the normal adrenal gland; this signals a possible role for miR-24 in adrenal pathophysiology.

In conclusion, I have, for the first time; demonstrated the direct involvement of miRNAs in the regulation of *CYP11B1* and *CYP11B2* expression. The discovery of this novel regulatory mechanism raises the possibility, in the longer term, of miRNA-based therapies for the treatment of aldosterone excess and essential hypertension. Such therapies permit the specific targeting and fine control of this system, leading to better management of hypertension and, therefore, reducing the incidence of cardiovascular disease.

8 Appendices

Table 8-7-1. Universal Probe Library qRT-PCR Assays.

| Gene | Forward Primer | Reverse Primer | UPL Probe # |
|----------|------------------------|-----------------------|-------------|
| B-actin | CCAACCGCGAGAAGATGA | CCAGAGGCGTACAGGGATAG | 64 |
| GAPDH | GCTCTCTGCTCCTCCTGTTC | ACGACCAAATCCGTTGACTC | 60 |
| StAR | TACGTGGCTACTCAGCATCG | ACCTGGTTGATGATGCTCTTG | 83 |
| CYP11A1 | AGGAGGGGTGGACACGAC | TTGCGTGCCATCTCATACA | 59 |
| CYP21A1 | GAGGGCACAGTCATCATTCC | GCTCCAGGAAGCGATCAG | 14 |
| CYP17A1 | CTATGCTCATCCCCACAG | TTGTCCACAGCAAACCTCACC | 67 |
| 3BHSDII | AGGCCTTCAGACCAGAATTG | CCTCAAGTACAGTCAGCTTGG | 50 |
| CYP11B1 | ACTAGGGCCCATTTCAGGT | GGCAGCATCACACACACC | 68 |
| CYP11B2 | GCACCTGCACCTGGAGATG | CACACACCATGCGTGGTCC | 57 |
| HSD11BI | CAATGGAAGCATTGTTGTTTCG | GGCAGCAACCATTGGATAAG | 20 |
| HSD11BII | GGGTCAAGGTCAGCATCATC | CACTGACCCACGTTTCTCAC | 71 |

The UPL Probe # refers to the specific fluorescent probe supplied in the Universal Probe Library Human Set (Roche Applied Sciences) which is unique to the amplicon.

Table 8-7-2. TaqMan® miRNA Assays

| Assay | miRNA Sequence | Product Code |
|-------------|---|--------------|
| miR-24 | UGGCUCAGUUCAGCAGGAACAG | 000402 |
| miR-21 | UAGCUUAUCAGACUGAUGUUGA | 000397 |
| miR-125a-5p | UCCCUGAGACCCUUAACCUGUGA | 002198 |
| miR-10b | UACCCUGUAGAACCGAAUUUGUG | 002218 |
| miR-143 | UGAGAUGAAGCACUGUAGCUC | 002249 |
| RNU48 | GATGACCCCAGGTAAGTCTGAG TGTGTCGCTGATGCCATCACCG CAGCGCTCTGACC | 001006 |

Table 8-7-3. miScript Primer Assays

| Assay | miRNA Sequence | Product Code |
|---------|------------------------|--------------|
| miR-24 | UGGCUCAGUUCAGCAGGAACAG | MS00006552 |
| SNORD48 | | MS00007511 |

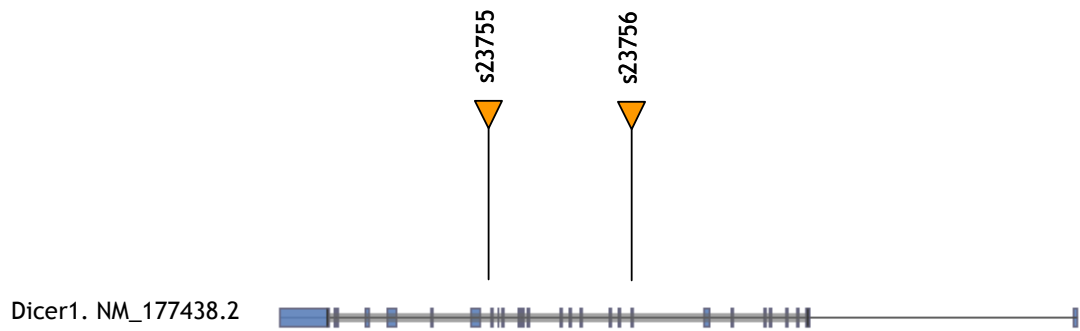


Figure 8-7-1. Location of Dicer1-Targeted siRNA Molecules.

Human *Dicer-1* mRNA structure; exons shown in blue boxes. s23755:siRNA A; s23756:siRNA B

9 References

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